Evaluation of new strategies to combat *Staphylococcus aureus* biofilm mediated infections in medical devices

Memoria presentada por

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para optar al grado de Doctor por la Universidad Pública de Navarra

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INFORMAN:

Que la presente memoria de Tesis Doctoral "**Evaluation of new strategies to combat *Staphylococcus aureus* biofilm mediated infections in medical devices**" elaborada por Doña **SAIOA BURGUI ERICE** ha sido realizada bajo su dirección y que cumple las condiciones exigidas por la legislación vigente para optar al grado de Doctor.

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Siguiendo los requisitos adoptados en el acuerdo A3/2015 del Comité de Dirección de la Escuela de Doctorado de Navarra para optar a la modalidad de tesis como compendio de publicaciones, los capítulos de esta Tesis Doctoral han sido publicados en:

1. **Valle J**, **Burgui S**, **Langheinrich D**, **Gil C**, **Solano C**, **Toledo-Arana A**, **Helbig R**, **Lasagni A**, **Lasa I**. 2015. Evaluation of Surface Microtopography Engineered by Direct Laser Interference for Bacterial Anti-Biofouling. Macromol Biosci **15**:1060–1069.

2. **Burgui S**, **Gil C**, **Solano C**, **Lasa I**, **Valle J**. 2018. A Systematic Evaluation of the Two-Component Systems Network Reveals That ArlRS Is a Key Regulator of Catheter Colonization by *Staphylococcus aureus*. Front Microbiol **9**:6069–11.

3. **Gil C, Solano C, Burgui S, Latasa C, García B, Toledo-Arana A, Lasa I, Valle J.** 2014. Biofilm matrix exoproteins induce a protective immune response against *Staphylococcus aureus* biofilm infection. Infect Immun **82**:1017–1029.

La contribución de **Saioa Burgui Erice** (SB) en estas publicaciones, es la siguiente:

1. Diseño del trabajo y preparación del manuscrito: JV, AL, IL
   Realización de los experimentos: JV, **SB**, LD, CG
   Preparación de los datos estadísticos y figuras: **SB**, ATA
   Escritura del manuscrito: JV, IL, CS

2. Diseño del trabajo y preparación del manuscrito: **SB**, CS, IL, and JV
   Realización de los experimentos: **SB**, CG, and JV
   Preparación de los datos estadísticos y figuras: **SB** and CG
   Escritura del manuscrito: JV, **SB**, IL, and CS

3. Diseño del trabajo y preparación del manuscrito: JV, IL
   Realización de los experimentos: CG, **SB**, CL, BG,
   Preparación de los datos estadísticos y figuras: CG, **SB**, ATA
   Escritura del manuscrito: JV, IL

Este trabajo ha sido realizado dentro de los siguientes proyectos de investigación:

- Título del proyecto: "Estudio de la formación de fibras amiloides como estructura para organizar la matriz de los biofilms bacterianos". SAF2015-74267-JIN. MINECO. Duración: 2017-2019

- Título del proyecto: "Descifrando las singularidades del exopolisacárido universal del biofilm (PNAG) y evaluación de su potencial biotecnológico". BIO2014-53530-R MINECO. Duración: 2015- 2017

- Título del proyecto: "Nuevas estrategias para el control de infecciones nosocomiales**".** RTC-2015-3184-1, MINECO. Duración: 2015-2017

- Título del proyecto: "Estudio funcional de la familia de proteínas Bap en las interacciones bacteria-bacteria y bacteria-huésped en la mastitis por estafilococos". AGL2011-23954, MINECO. Duración: 2012-2015

- Título del proyecto: "Estudio de la transferencia horizontal de genes y de la formación de biofilm a nivel de célula individual en *Staphylococcus aureus*". BIO2011-30503-C02-02. Duración: 2012-2014

- Título del proyecto: Desarrollo y Evaluación de las propiedades antibacterianas de superficies con relieves nanoestructurados generados por "direct laser interference pattering". IIQ14066.RI. Gobierno de Navarra. Duración: 2010-2014.

RESUMEN

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Según recoge el estudio EPINE-EPS orientado a la recogida de datos de prevalencia de las infecciones nosocomiales en España, en el año 2017 alrededor de 62.000 pacientes adquirieron algún tipo de infección nosocomial durante su estancia en un centro hospitalario. La práctica médica actual resulta impensable sin la utilización de distintos dispositivos implantables tales como válvulas, catéteres venosos centrales, catéteres urinarios o prótesis articulares para el tratamiento de los pacientes. A pesar de todos sus beneficios, un aspecto negativo asociado a la utilización de dispositivos médicos invasivos es un mayor riesgo a sufrir infecciones por microorganismos que crecen adheridos a su superficie. Las infecciones relacionadas con dispositivos médicos suponen un porcentaje creciente y significativo de las infecciones nosocomiales, y provocan un incremento del gasto sanitario, así como una mayor morbilidad y mortalidad del paciente.

Aunque, una gran variedad de microorganismos puede causar infecciones asociadas a implantes, *Staphylococcus aureus* y *S. epidermidis* ocupan un puesto muy destacado entre los agentes que con mayor frecuencia causan infecciones asociadas a dispositivos médicos. Su presencia en la piel humana facilita sus posibilidades de alcanzar la superficie del implante y por otro lado su elevada capacidad para adherirse a la superficie de materiales abióticos les permite adherirse irreversiblemente a su superficie. Una vez que la bacteria se ha adherido a la superficie, las bacterias comienzan a dividirse y secretar una matriz extracelular que las rodea formando lo que comúnmente se conoce como biofilm. La formación de biofilm incrementa la resistencia de las bacterias a los tratamientos antibióticos y a la acción del sistema inmune. En el caso de las infecciones producidas por *S. aureus* esta situación se agrava por la existencia de cepas resistentes a múltiples antibióticos, como meticilina y antibióticos glucopéptidos.

En esta tesis hemos trabajado en distintas estrategias que podrían ayudar a reducir la incidencia de estas infecciones. En el primer capítulo, hemos estudiado cómo la modificación de la topografía de la superficie del biomaterial del implante médico puede reducir la adhesión bacteriana y la formación de biofilms. Para ello, hemos utilizado una metodología laser de interferencia directa (DLIP) para modificar la topografía de la superficie de poliestireno a escala submicrométrica. Los resultados han revelado que las estructuras micrométricas tridimensionales tienen un profundo impacto sobre la adhesión bacteriana. Los patrones tipo línea y pilar mejoran la

adhesión de *S. aureus*, mientras que una microtopografía laminar irregular reduce la adhesión de *S. aureus* tanto en condiciones de cultivo estático, como de flujo continuo. Además, las superficies laminares mantienen la capacidad de inhibir la adhesión de *S. aureus* tanto cuando la superficie se cubre de proteínas del suero humano tras su implantación.

En el segundo capítulo, nos hemos interesado en estudiar el papel que juegan los sistemas de dos componentes (TCS) de *S. aureus* en su adaptación para colonizar y sobrevivir en la superficie de los implantes médicos. Utilizando un modelo murino de infección por catéter *in vivo* y una colección de mutantes en cada uno de los TCS no esencial de *S. aureus*, investigamos el requerimiento de cada TCS para colonizar el catéter implantado. Entre los 15 mutantes en TCS no esenciales, el mutante *arlRS* ha exhibido la deficiencia más importante en su capacidad para colonizar catéteres implantados. Además, el mutante *arlRS* ha sido el único que ha presentado un déficit importante en la producción de PNAG, el principal exopolisacárido de la matriz del biofilm de *S. aureus* cuya síntesis está mediada por el locus *icaADBC*. Nuestros resultados indican que la regulación de la síntesis de PNAG por ArlRS se produce a través de la represión de IcaR, un represor transcripcional de la expresión del operón *icaADBC*. Así, la deficiencia en la colonización del catéter se restauraba cuando el mutante *arlRS* se complementó con el operón *icaADBC*. Estos resultados indican que ArlRS es un TCS clave para la formación de biofilm en la superficie de los catéteres implantados y que la activación de la producción del exopolisacáridos PNAG es, entre los muchos rasgos controlados por el sistema ArlRS, uno de los que mas contribuyen a la colonización del catéter.

Por último, en el tercer capítulo abordamos la prevención de la formación de biofilm de *S. aureus* mediante el desarrollo de vacunas antibiofilm. Bajo la premisa de que en una infección causada por bacterias creciendo en biofilm, la interfaz entre el huésped y la bacteria es la matriz extracelular, analizamos el potencial de proteínas extracelulares secretadas a la matriz del biofilm para inducir una respuesta inmune protectora contra infecciones por *S. aureus*. Mediante el uso de técnicas proteómicas, caracterizamos los exoproteomas de la matriz del biofilm producido por dos cepas clínicas de *S. aureus* que producen biofilms de naturaleza exopolisacáridica o proteica. Los resultados han mostrado que con independencia de la naturaleza de la matriz del biofilm, existe un núcleo común de proteínas secretadas a la matriz de ambos tipos de biofilms. La inmunización con un extracto de exoproteínas de la matriz del biofilm induce una respuesta inmune humoral y la producción de interleuquinas IL-10 e IL-17

en un modelo de infección de ratón. Los anticuerpos producidos promueven la opsonofagocitosis y la muerte de *S. aureus*. Como consecuencia de la inducción del sistema inmune, los ratones inmunizados presentaban un recuento significativamente menor de bacterias en la superficie del implante y en el tejido circundante utilizando un modelo de infección con malla intraperitoneal. En conjunto, los datos de este trabajo muestran el potencial que las exoproteínas de la matriz del biofilm pueden tener como vacuna multivalente frente a infecciones causadas por biofilms de *S. aureus*.

ABSTRACT

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According to the EPINE-EPS study that collects data on the prevalence of nosocomial infections in Spain, in the year 2017, around 62,000 patients develop some type of nosocomial infection during their stay in a hospital center. Current medical practice is unthinkable without the use of different implantable devices such as valves, central venous catheters, urinary catheters or joint prostheses for the treatment of patients. Despite all their benefits, a negative aspect associated with the use of invasive medical devices is an increased risk of infections by microorganisms that grow attached to their surfaces. Infections related to medical devices represent a growing and significant percentage of nosocomial infections, and cause an increase in health care costs, as well as a higher morbidity and mortality.

Although a large variety of microorganisms can cause infections associated with implants, *Staphylococcus aureus* and *S. epidermidis* occupy a very prominent position among the agents that most frequently cause infections associated with medical devices. Their presence on human skin facilitates the chances of reaching the surface of the implant and on the other hand, their high capacity to adhere to the surface of abiotic materials allows them to adhere irreversibly to surfaces. Once bacteria have adhered to the surface, they begin to divide and secrete an extracellular matrix that surrounds them forming what is commonly known as a biofilm. The formation of a biofilm increases the resistance of bacteria to antibiotic treatments and to the action of the immune system. In the case of infections caused by S. aureus this situation is aggravated by the existence of strains resistant to multiple antibiotics, such as methicillin and glycopeptide antibiotics.

In this thesis we have worked on different strategies that could help reduce the incidence of these infections. In the first chapter, we studied how modifying the topography of the biomaterial surface of the medical implant can reduce bacterial adhesion and biofilm formation. For this, we used a direct laser interference patterning methodology (DLIP) to modify the topography of polystyrene surfaces at submicrometric scale. The results revealed that three-dimensional micrometric structures have a profound impact on bacterial adhesion. The line and pillars patterns improve the adhesion of *S. aureus*, while irregular laminar microtopography reduces the adhesion of *S. aureus* under both static and continuous flow conditions. This reduction occurs in the absence and presence of human serum proteins. In addition, in a murine model of biofilm formation on materials implanted intraperitoneally, the

surface with laminar topography inhibits the adhesion of *S. aureus* both when contamination occurs on the naked material prior to its implantation, and when the infection is generated after implantation of the surface.

In the second chapter, we were interested in studying the role played by the two-component systems (TCS) of *S. aureus* in its adaptation to colonize and survive on the surface of medical implants. Using a *in vivo* murine catheter infection model and a collection of mutants in each non-essential TCS of *S. aureus*, we investigated the requirement of each TCS to colonize the implanted catheter. Results showed that, among the 15 mutants in non-essential TCS, the *arlRS* mutant exhibits the highest deficiency in its ability to colonize implanted catheters. In addition, the *arlRS* mutant is the only one that presents a significant deficit in the production of PNAG, the main exopolysaccharide of the *S. aureus* biofilm matrix, whose synthesis is mediated by the *icaADBC* locus. Our results also indicate that the regulation of PNAG synthesis by ArlRS occurs through the repression of IcaR, a transcriptional repressor of the expression of the *icaADBC* operon. Thus, the deficiency in colonization of the catheter is restored when the *arlRS* mutant is complemented with the *icaADBC* operon. These results indicate that ArlRS is a key TCS for the formation of a biofilm on the surface of implanted catheters and that the activation of PNAG exopolysaccharide production is, among the many traits controlled by the ArlRS system, a major contributor to colonization of catheters.

Finally, in the third chapter we addressed the prevention of *S. aureus* biofilm formation through the development of antibiofilm vaccines. Under the premise that in an infection caused by bacteria growing inside a biofilm, the interface between the host and the bacterium is the extracellular matrix, we analyzed the potential of extracellular proteins secreted to the biofilm matrix to induce a protective immune response against *S. aureus* infections. Through the use of proteomic techniques, we characterized the exoproteome of the biofilm matrix produced by two *S. aureus* clinical strains that produce biofilms of exopolysaccharidic or proteinaceous nature. Results showed that regardless of the biofilm matrix nature, there is a common nucleus of proteins secreted into the matrix. Immunization with an exoprotein extract from the biofilm matrix induces a humoral immune response and the production of IL-10 and IL-17 interleukins in a mouse infection model. The antibodies produced promote opsonophagocytosis and the death of *S. aureus*. As a result of the induction of the immune system, immunized mice have a significantly lower bacterial count on an implant surface and in the surrounding tissue, using an intraperitoneal mesh infection

model. Taken together, the data from this work show the potential that the exoproteins of the biofilm matrix can have as a multivalent vaccine against infections caused by *S. aureus* biofilms.

INTRODUCCIÓN

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1. Infecciones asociadas a dispositivos médicos

1.1. Infecciones nosocomiales

Las infecciones nosocomiales o intrahospitalarias son aquellas infecciones que el paciente adquiere durante el ingreso a una institución sanitaria, mientras recibe tratamiento para alguna condición médica o quirúrgica y en quien la infección no se había manifestado ni estaba en período de incubación. Se asocian con varias causas, incluyendo, complicaciones postquirúrgicas, transmisión entre pacientes y trabajadores de la salud, principalmente en el caso de pacientes inmunocomprometidos o como resultado de un consumo frecuente de antibióticos (Allegranzi *et al.*, 2007). Las tasas más altas de infección se encuentran en las unidades de cuidados intensivos (UCI) de adultos y pediátricas. En algunos casos estas infecciones pueden ser motivo de ingreso en la UCI, mientras que en otros casos son consecuencia de la estancia en las mismas y conllevan un destacado impacto en términos de mortalidad y costes hospitalarios. En su conjunto, se ha estimado que las infecciones nosocomiales son causa directa de más de 175.000 muertes en Europa y 90.000 en Estados Unidos cada año, y suponen un gasto para los sistemas de la Salud de miles de millones de euros (Guggenbichler *et al.*, 2011).

1.2. Infecciones nosocomiales asociadas a dispositivos médicos

Un porcentaje muy elevado de las infecciones nosocomiales están asociadas a la utilización de dispositivos médicos invasivos (Whitehouse *et al.*, 2002). Algunos ejemplos de dispositivos invasivos son los instrumentos de diagnóstico y terapéuticos utilizados en otorrinolaringología, oftalmología, odontología y ginecología, catéteres urinarios, catéteres cardiovasculares, catéteres neurológicos, tubos endotraqueales, ventiladores mecánicos, prótesis, válvulas (**Tabla 1**).

Tabla 1. Tipos de dispositivos médicos invasivos

Intravascular
Catéter de corta duración - Central (tunelado/no tunelado) - Periférico (venosos/arterial) Catéter de larga duración - Hickman - Reservorio - Picc
Cardiovascular
Válvula mecánica Desfibrilador implantable Injerto vascular Dispositivo asistencia ventricular Stent coronario Monitor implantable
Neurocirugía
Derivación ventriculoperitoneal Reservorio de Ommaya Dispositivos de presión intracraneal Simulador neurológico implantable
Ortopédico
Implantes articulares Implantes reconstrucción ortopédica Implantes espinales Dispositivos fijación de fracturas
Urológicos
Implantes de pene
Ginecológicos
Implantes de pecho
Otorrinolaringología
Implantes cocleares Implantes del oído medio
Oftalmología
Lentes intraoculares Tubos glaucoma
Odontología
Implante dental

De entre todos los dispositivos médicos invasivos, los catéteres y biomateriales implantables son los que con mayor frecuencia causan infecciones nosocomiales. En EEUU se utilizan anualmente más de 150 millones de dispositivos intravasculares para la administración de fluidos, fármacos, hemoderivados, nutrición parenteral, siendo la mayoría catéteres venosos periféricos, y más de 5 millones de catéteres venosos centrales (CVC) (Mermel, 2000). Siguiendo esta misma tendencia, la utilización de CVC ha aumentado del 3 al 6 % CVC en los hospitales españoles en los últimos quince años.

En el caso de los biomateriales implantables solo en los Estados Unidos, se implantaron 332.000 prótesis de caderas y 719.000 artroplastias de rodilla en el año 2010. Se calcula que para el año 2030 los implantes de cadera llegarán a 572.000 y se realizarán 3,48 millones de implantes de rodilla (Kurtz *et al.*, 2007). En nuestro país, en el año 2004 se realizaron 85 reemplazos de cadera y 75 de rodilla por cada 100.000 habitantes. Estas cifras aumentaron a 110 y 125 reemplazos respectivamente en 2014. Si nos fijamos en Navarra **(Tabla 2)**; según los datos recogidos por el servicio de traumatología del Complejo Hospitalario de Navarra durante el año 2016, se realizaron casi 1600 implantes primarios de prótesis de cadera. Este dato señala que en nuestra comunidad y según el censo de la Comunidad Foral a día 1 de enero de 2017, tenemos una ratio de implantación de prótesis de cadera de una por cada 400 habitantes.

Tabla 2. Implantes de cadera realizados en los diferentes hospitales de Navarra en 2016

	PAMPLONA	ESTELLA	TUDELA	TOTAL
Enero	73	7	16	**96**
Febrero	141	8	26	**175**
Marzo	141	12	23	**176**
Abril	126	12	22	**160**
Mayo	126	11	24	**161**
Junio	120	16	21	**157**
Julio	63	3	8	**74**
Agosto	60	8	7	**75**
Septiembre	102	19	17	**138**
Octubre	118	12	9	**139**
Noviembre	112	13	24	**149**
Diciembre	85	7	7	**99**
TOTAL	**1267**	**128**	**204**	**1599**

Según el estudio EPINE-EPPS de 2017, orientado a la recogida de datos de prevalencia de las infecciones nosocomiales en España y que incluye datos de 313 hospitales españoles y 61.673 pacientes, la incidencia de infección asociada a catéteres oscila entre 0,1 y 7 episodios por cada 100 catéteres **(Tabla 3)**. En el caso de los dispositivos implantables, las tasas de infección varían del 1 a 5%, dependiendo del tipo del implante. En el caso de las válvulas protésicas, las tasas de infección varían del 1 al 5%, presentando una tasa de mortalidad muy elevada. Las infecciones se presentan en menos del 2% de los casos en prótesis articulares. Se ha visto además que las tasas de infección suben muy significativamente, alrededor del 40%, después de una reinfección en procedimientos de reemplazo (Corvec *et al.*, 2012).

Tabla 3. Infecciones asociadas a dispositivos médicos (análisis EPINE-EPPS 2017)

Implante	nº/año	Infecciones	Mortalidad
Catéter intravascular	200.000.000	< 0,1-7%	15-20%
Sonda uretral	500.0000	5-10%	
Shunts LCR	80.000	10-15%	
Marcapasos	60.000	0-3%	2%
Válvulas protésicas	100.000	1-5%	34%
Prótesis articulares	350.000	<1-2%	2,5%
Implantes dentales	436.000	0	

Por ejemplo, en los datos recogidos en el Complejo Hospitalario de Navarra para los implantes de cadera realizados en esta comunidad en el año 2016 reflejan que un 3,88% de las prótesis implantadas resultaron infectadas y que el 63% de los pacientes con una infección de la prótesis tuvieron que volver a ser ingresados por una reinfección.

1.3. Consecuencias clínicas y económicas de las infecciones asociadas a dispositivos médicos

En 2014, el precio medio estimado de un reemplazo de cadera en los países de la UE fue de aproximadamente 5.600 euros, mientras que el precio de un reemplazo de rodilla se encontraba en el mismo rango costando alrededor de 5.700 euros (OECD, 2016). En el caso de infección, un número elevado de estos pacientes requiere la retirada del implante y un prolongado tratamiento antibiótico antes de que se le pueda

volver a implantar la prótesis. Al gasto de la intervención de reemplazo, habría que añadir los costes indirectos provocados por la inmovilidad de los pacientes convalecientes y el coste de los tratamientos con antibióticos prolongados que se suman a la cirugía de reemplazo (Tande and Patel, 2014). Finalmente, es importante destacar que las infecciones asociadas a dispositivos médicos pueden provocar en muchos casos la muerte del paciente. Según el estudio EPINE-EPPS de 2017 la mortalidad atribuible a la infección asociada al uso de dispositivos médicos invasivos (catéteres intravasculares) varía entre el 2 y 30%.

1.4. Etiología de la infección y agentes causales

Muchas infecciones asociadas a dispositivos médicos ocurren en el entorno nosocomial como resultado de la contaminación de los dispositivos desde la microbiota de la piel y mucosas de los pacientes o del personal hospitalario. La infección del material implantado generalmente se produce durante la colocación del dispositivo. Otros mecanismos patogénicos menos frecuentes incluyen, la colocación de un dispositivo ya contaminado, la contaminación durante la cirugía, las infecciones por contigüidad y por diseminación hematógena desde un foco distante (Murdoch *et al.*, 2001).

En términos generales, las bacterias residentes en la piel y las mucosas como los estafilococos, y en especial las especies coagulasa negativas (ECN) y *Staphylococcus aureus* son los agentes etiológicos más frecuentes de las infecciones relacionadas con dispositivos médicos. En un estudio epidemiológico sobre 309 pacientes con infecciones ortopédicas tratadas entre 2011 y 2014 en el Instituto Ortopédico Rizzoli (Yousif *et al.*, 2015), *S. aureus* resultó ser siempre el principal agente etiológico en todos los tipos de infecciones estudiados.

Otros agentes etiológicos asociados con menor frecuencia incluyen enterococos, bacilos gram negativos (enterobacterias, *Pseudomonas aeruginosa* y otros no fermentadores), y levaduras (*Candida albicans, C. glabrata, C. parapsilosis*). En las infecciones hematógenas el origen puede ser cutáneo (*Streptococcus pyogenes*), bucodental (estreptococos grupo viridans y anaerobios) o genitourinario (bacilos gram negativos, enterococos) (Eiff *et al.*, 1999; Donlan, 2001; Götz, 2002; Hugonnet *et al.*, 2004; Eiff *et al.*, 2005; Arciola *et al.*, 2005; Kathju *et al.*, 2009). Las bacterias que generalmente se aíslan de los catéteres urinarios son principalmente, *E. faecalis*, *E. coli* y *Proteus mirabilis*. En las neumonías asociadas al uso de ventiladores en pacientes con

respiración asistida al menos el 20% de los casos están causadas por *S. aureus* seguido de *P. aeruginosa* (Chastre and Fagon, 2002).

Tabla 4. Agentes patógenos asociados a infecciones en dispositivos médicos

Dispositivo	Patógeno prevalente	
	Principal	Secundario
Catetér venoso central	**CoNS**	*S. aureus*, enterococci, *Candida* spp., *K. pneumoniae, P. aeruginosa*
Catéter urinario	***E. coli***	*Candida* spp., CoNS, *E. faecalis, P. mirabilis*
Válvula mecánica cardiaca	**CoNS**	*S. aureus, Streptococcus* spp., GNB, enterococci, diptheriosis
Dispositivo asistencia ventricular	**CoNS**	*S. aureus, Candida* spp., *P. aeruginosa*
Stent coronario	***S. aureus***	CoNS, *P. aeruginosa, Candida* spp.
Derivación ventricular neuroquirúrgica	***Staphylococci***	*Streptococcus* spp., *Corynebacterium*, GNB
Catéter de diálisis peritoneal	***S. aureus***	*P. aeruginosa*, otras Gram-negativas spp., *Candida* spp.
Prótesis ortopédica	***Staphylococci***	*S. pneumoniae*, *Streptococcus* spp., *P.acnes*
Dispositivos de fijación de fracturas	**CoNS**	*S. aureus*, *Propionibacterium* spp., *Corynebacterium*
Tubos endotraqueales	**Bacilo gram negativo enterico**	*P. aeruginosa*, *Streptococcus* spp., *Staphylococcus* spp.
Implantes de pene	**CoNS**	*S. aureus*, enteric GNB, *P. aeruginosa*, *Serratia* spp., fungi
Implantes de pecho	***Staphylococci***	*E. coli*, *peptostreptococci*, *C. perfringens, P.acnes*
Implantes cocleares	***S. aureus***	*P. aeruginosa, Streptococcus* spp., *N. meningitidis*, fungi

2. *Staphylococcus aureus*

2.1. Patogenia de *S. aureus*

S. aureus es una bacteria gram-positiva ubicua y altamente versátil capaz de adaptarse a diferentes tipos de ambientes. Reside principalmente en la piel y el tracto respiratorio, pudiendo vivir como un organismo comensal o como un patógeno (Salyers *et al.*, 2002). Se estima que aproximadamente el 20% de los adultos son portadores nasales persistentes de la bacteria (Wertheim *et al.*, 2005) y 60% son portadores de *S. aureus* de manera intermitente (Kluytmans *et al.*, 1997). *S. aureus* es inofensivo en estos lugares, pero se convierte en un patógeno extremadamente peligroso cuando atraviesa la barrera del epitelio y accede a los tejidos internos desde donde puede infectar casi cualquier órgano y causar un amplio espectro de infecciones que incluyen abscesos, neumonía, endocarditis, osteomielitis, sepsis e infecciones asociadas con implantes

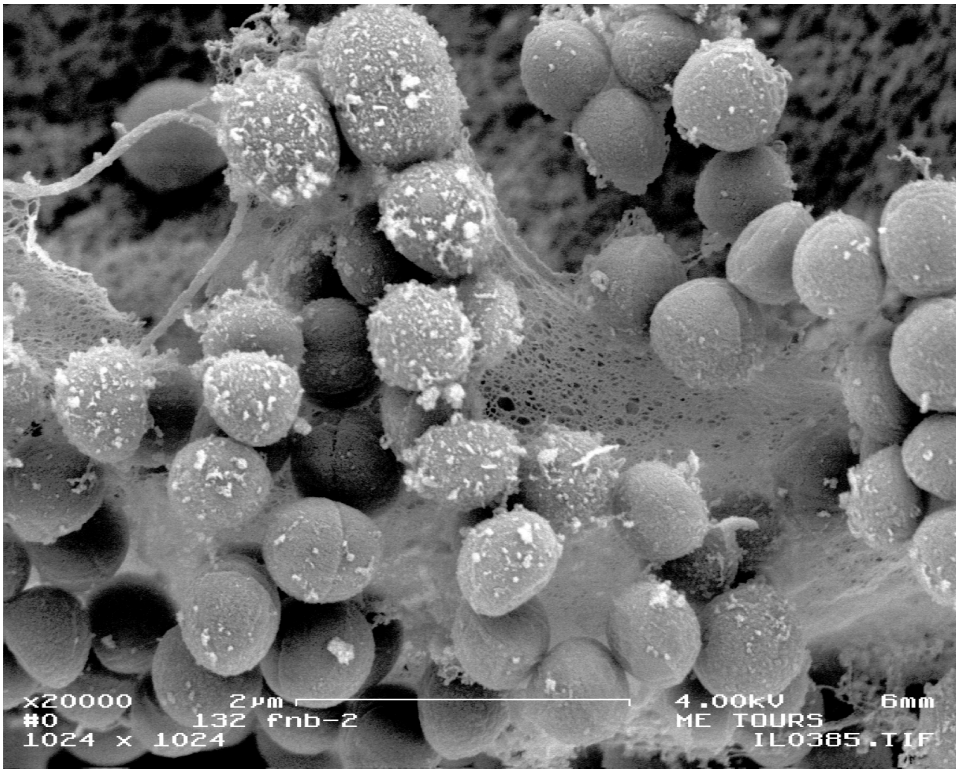
médicos (Gordon and Lowy, 2008). Las infecciones por *S. aureus* son la principal causa de infecciones nosocomiales en todo el mundo, especialmente entre pacientes inmunodeprimidos (Julie A Wu *et al.*, 2003; Yarwood *et al.*, 2004). El tratamiento de las infecciones producidas por *S. aureus* se ha agravado en los últimos años por la existencia de cepas resistentes a múltiples antibióticos, denominadas *S. aureus* resistentes a la meticilina (MRSA) (Kennedy *et al.*, 2008). Estas cepas son únicamente sensibles a los antibióticos glucopéptidos como vancomicina y teicoplanina. Sin embargo, en los últimos años, también han sido aisladas cepas de *S. aureus* resistentes a la vancomicina, probablemente debido al uso creciente de este antibiótico para tratar infecciones asociadas a MRSA (Jones, 2008).

La patogenicidad de *S. aureus* depende de la acción combinada de sus múltiples factores de virulencia como proteínas de superficie, superantígenos, toxinas y enzimas, como proteasas, lipasas y elastasas (Gordon and Lowy, 2008). *S. aureus* ha desarrollado la capacidad de evadir el sistema inmune del huésped. Para ello, secreta proteínas que inhiben la activación del complemento y la quimiotaxis de neutrófilos. Puede lisar neutrófilos, neutralizar antimicrobianos y péptidos bactericidas o modificar su superficie celular para reducir su efectividad. Además, puede expresar polisacáridos y proteínas que inhiben la opsonización por anticuerpos y complemento, sobrevivir en fagosomas, y su pared celular es resistente a la lisozima. Por otro lado, la expresión de superantígenos evita la respuesta inmune humoral normal dando como resultado anergia e inmunosupresión (Timothy J Foster, 2005). Finalmente, la capacidad de *S. aureus* para formar y residir en biofilms también constituye un importante factor de virulencia que aumenta su persistencia y nivel de resistencia a los antimicrobianos, mejorando aún más su patogenicidad (Brady *et al.*, 2008).

2.2. El biofilm como factor de patogenicidad en *S. aureus*

Pacientes con dispositivos médicos permanentes, como catéteres, válvulas cardíacas, prótesis, etc., pueden desarrollar fácilmente infecciones estafilocócicas, debido a la capacidad de *S. aureus* para colonizar la superficie del implante y formar biofilm (**Figura 1)** (Costerton *et al.*, 1995). Los biofilms son comunidades de bacterias adheridas a superficies (inertes o tejido vivo) y englobadas en una matriz extracelular que las propias bacterias sintetizan (Costerton *et al.*, 1999). El interior del biofilm constituye un ambiente protegido que permite a *S. aureus* sobrevivir en un ambiente hostil.

Figura 1. Biofilm formado por S. aureus sobre una superficie de poliestireno



Estas son algunas de las ventajas que han sido atribuidas al crecimiento en biofilm:

- El biofilm facilita la colonización de superficies. La formación del biofilm permite el establecimiento de *S. aureus* sobre superficies inertes, o tejidos vivos. La íntima adhesión de las bacterias entre si y con la superficie les protege frente a la eliminación por el arrastre mecánico al que están expuestas en algunos hábitats como catéteres en vasos sanguíneos, tubos de diálisis, materiales implantables etc.

- El biofilm protege a *S. aureus* frente al sistema inmune del hospedador. El biofilm confiere resistencia frente a la fagocitosis y a la opsonización mediada por el complemento (McKenney *et al.*, 1998; McKenney *et al.*, 1999; Barrio *et al.*, 2000). Además, el biofilm protege frente a la destrucción oxidativa y frente a la acción de péptidos catiónicos, debido a que estas moléculas difunden con gran dificultad a través del entramado de la matriz del biofilm (Kraus and Peschel, 2008).

- El biofilm aumenta la resistencia a los antibióticos. Numerosos estudios han demostrado que *S. aureus* es menos susceptible al tratamiento antibiótico cuando crecen en forma de biofilm (Donlan and Costerton, 2002; Cafiso *et al.*, 2004; Høiby *et al.*, 2010; Kostakioti *et al.*, 2013). Existen varias hipótesis que explican la mayor resistencia al tratamiento antibiótico: i) la matriz extracelular constituye una barrera física y química que dificulta la penetración de los antibióticos en el interior del biofilm; ii) las bacterias en el interior del biofilm presentan una menor tasa de crecimiento; iii) el estado fisiológico latente que presentan las bacterias en el biofilm les hace ser menos susceptibles a los antibióticos; y iv) En el biofilm se crean microambientes específicos que antagonizan la acción de los antibióticos (O'Toole *et al.*, 2000).

En el proceso de formación del biofilm se distinguen 3 etapas bien diferenciadas:

1) Adherencia primaria: las bacterias se adhieren irreversiblemente a la superficie.
2) Adherencia intercelular: en esta fase las bacterias proliferan y se acumulan en grupos de células de múltiples capas y sintetizan la matriz extracelular que les engloba.
3) Dispersión: algunas bacterias se liberan del biofilm y pueden dispersarse a otros sitios a través de la linfa y el torrente sanguíneo y colonizar nuevas superficies. Como consecuencia, las bacterias liberadas pueden establecer infecciones secundarias (Otto, 2013).

2.2.1 Factores implicados en la adhesión primaria

La unión inicial a la superficie puede implicar interacciones tanto específicas como inespecíficas con la superficie que se coloniza. Las interacciones no específicas incluyen la hidrofobicidad o la carga de la superficie celular. Los factores de la superficie de la bacteria que pueden afectar a la adhesión primaria son:

- Ácidos teicoicos

Los ácidos teicoicos (AT) son polímeros de pared celular altamente cargados negativamente que se unen covalentemente al péptidoglicano (ácidos teicoicos) o anclados a la membrana (ácidos lipoteicoicos). Tienen un contenido variable de sustituciones de ésteres de D-alanina que reducen su carga neta negativa mediante la

introducción de grupos amino básicos (Neuhaus y Baddiley, 2003). Se ha estudiado que una mutación en el gen *dltA,* que afecta la incorporación de ésteres D-alanina en ácidos teicoicos, aumenta la carga neta negativa en la superficie de *S. aureus*, lo que resulta en una reducida capacidad para colonizar y formar biofilms en superficies de plástico y vidrio. Este fenotipo se atribuye a una falta en la adhesión primaria ya que las interacciones intercelulares no se vieron afectadas en el mutante (Gross et al., 2001).

- Autolisinas

La autolisina AtlA de *S. aureus,* (Oshida *et al.*, 1995), al igual que la autolisina AtlE de *S. epidermidis* (Heilmann *et al.*, 1997), es una enzima bifuncional con una actividad amidasa y otra glucosaminidasa y está involucra en el recambio de la pared celular, la división y separación celular y en la lisis de las bacterias inducidas por los antibióticos β-lactámicos (Biswas *et al.*, 2006). La autolisina AtlA, junto con los ácidos teicoicos influyen en la hidrofobicidad de la superficie bacteriana y, por ello, se han implicado en la adhesión primaria de *S. aureus* a las superficies. Se ha demostrado que los mutantes en autolisinas presentan reducida la adhesión primaria a superficies y forman un biofilm defectuoso (Sugai *et al.*, 1995; Heilmann *et al.*, 1997; Biswas *et al.*, 2006; Houston *et al.*, 2011). AtlE posee además una actividad de unión a vitronectina y fibronectina, lo que indica que AtlE desempeña un papel en la colonización no solo de las superficies poliméricas sino también superficies recubiertas con proteínas del plasma (Heilmann *et al.*, 1997).

En el caso de las interacciones especificas, los factores implicados en la adhesión de *S. aureus* son:

- Componentes de la superficie microbiana que reconocen moléculas de la matriz adhesiva (MSCRAMMs):

La capacidad de *S. aureus* para adherirse a los componentes de la matriz extracelular y a las proteínas plasmáticas constituye un factor crucial en el proceso de colonización. La adherencia es frecuentemente mediada por proteínas de la familia de MSCRAMMs (Microbial Surface Components Recognising Adhesive Matrix Molecules). Estas proteínas están ancladas covalentemente a la pared celular de la bacteria a través del motivo LPXTG (Patti *et al.*, 1994; T J Foster and Höök, 1998; Clarke and Simon J Foster, 2006). Las MSCRAMMs pueden mediar la adherencia de *S. aureus* a superficies abióticas una vez que proteínas del plasma, tales como fibronectina o fibrinógeno, han

cubierto la superficie del dispositivo (Lopes *et al.*, 1985; Chhatwal and Blobel, 1987; Delmi *et al.*, 1994; Sinha *et al.*, 1999). Algunas de las proteínas MSCRAMMs más importantes en este proceso son las proteínas de unión a fibronectina A y B (FnBPA, FnBPB) (Flock *et al.*, 1987; Jönsson *et al.*, 1991), la proteína de unión al colágeno (Cna) (Switalski *et al.*, 1993), las proteínas de unión al fibrinógeno, y las proteínas de agregación A (ClfA) (McDevitt *et al.*, 1994) y B (ClfB) (Abraham and Jefferson, 2012).

- Adhesinas secretadas (SERAM)

S. aureus también produce adhesinas que son secretadas y se denominan en conjunto SERAMs (Secreteable expanded Repertoire Adhesive Molecules). Estas proteinas facilitan la unión de *S. aureus* a células eucariotas, plaquetas, proteínas de la matriz extracelular y superficies inertes (Chavakis *et al.*, 2005). Entre ellas, destacan la proteína de adherencia extracelular (Eap), la proteína de unión al fibrinógeno extracelular (Efb), la proteína de unión a la matriz extracelular (Emp) y la proteína de unión al factor von Willebrand (vWbp). Se ha demostrado que las proteínas Emp y Eap desempeñan un papel importante en la formación de biofilms de *S. aureus* en condiciones ambientales con bajo contenido en hierro (Johnson *et al.*, 2008).

2.2.2. Factores de adhesión intercelular

- Exopolisacárido PIA/PNAG

El Polisacárido de Adhesión Intercelular (PIA) o poli-N-acetilglucosamina (PNAG), es uno de los componentes más importantes que intervienen en adhesión intercelular en el proceso de formación de biofilm de *S. aureus*. Es un polisacárido formado por monómeros de N-acetilglucosamina unidos por enlace β-1-6 (poli-β-1,6-GlcNAc). Fue purificado del biofilm de una cepa de *S. epidermidis* e inicialmente se le denominó PIA (polysaccharide intercellular adhesin) (Tojo *et al.*, 1988; Christensen *et al.*, 1990; Mack *et al.*, 1994; Mack *et al.*, 1996; Baldassarri *et al.*, 1996). Posteriormente fue descrito en cepas de *S. aureus* y en base a su composición se le denominó PNAG (McKenney *et al.*, 1999; Maira-Litràn *et al.*, 2002). La síntesis de PIA/PNAG depende de la actividad de 4 proteínas codificadas por el operón *icaADBC* (Mack *et al.*, 1996; Cramton *et al.*, 1999). La enzima IcaA tiene actividad N-acetil-glucosaminil transferasa y requiere del producto del gen *icaD* para su actividad óptima. El gen *icaC* codifica para una proteína de membrana que alarga las cadenas de oligómeros que se han sintetizado y permite su translocación a la superficie de la bacteria (Gerke *et al.*, 1998). IcaB es una

proteína anclada a la superficie implicada en la deacetilación del polisacárido (Maira-Litràn *et al.*, 2002; Vuong, Kocianova, *et al.*, 2004).

Aunque la mayoría de los aislados de *S. aureus* contienen el operón *ica* (Cramton *et al.*, 1999; Fowler *et al.*, 2001; Arciola *et al.*, 2001; Martín-López *et al.*, 2002; Fitzpatrick *et al.*, 2005), en condiciones de laboratorio solo unos pocos lo expresan y forman biofilms. Esto sugiere que la expresión del operón *ica* está estrechamente controlada y sujeta a regulación ambiental. Así, se ha descrito que estímulos como alta osmolaridad, condiciones anaeróbicas, la temperatura y concentraciones subinhibitorias de ciertos antibióticos inducen la transcripción de los genes *ica* y la formación de biofilms (Rachid, Ohlsen, Wallner, *et al.*, 2000; Rachid, Ohlsen, Witte, *et al.*, 2000; Cramton *et al.*, 2001). Diversos estudios muestran que la producción de PIA/PNAG también se induce *in vivo* (McKenney *et al.*, 1999; Fluckiger *et al.*, 2005). Sin embargo, la función de este exopolisacárido en el proceso de infección sigue siendo controvertido. Algunos estudios coinciden en que este polisacárido confiere protección frente los principales componentes del sistema inmune innato (Vuong, Voyich, *et al.*, 2004; Vuong, Kocianova, *et al.*, 2004; Cerca *et al.*, 2007). Sin embargo, resultados de otros trabajos sugieren que los mutantes en el operón *ica* son tan virulentos como sus cepas parentales isogénicas (Francois *et al.*, 2003; Kristian *et al.*, 2004; Beenken *et al.*, 2004; Fluckiger *et al.*, 2005; Vergara-Irigaray *et al.*, 2008; Merino *et al.*, 2009).

Una idea ampliamente extendida en el ámbito de los biofilm bacterianos es que la matriz extracelular del biofilm esta mayoritariamente compuesta por exopolisacáridos. Sin embargo, el análisis de la matriz del biofilm de *S. aureus* ha mostrado que la matriz del biofilm puede tener naturaleza proteica (Cucarella *et al.*, 2001; Beenken *et al.*, 2004; Rohde *et al.*, 2005; Fitzpatrick *et al.*, 2005; Toledo-Arana *et al.*, 2005; Kogan *et al.*, 2006; O'Neill *et al.*, 2007; Corrigan *et al.*, 2007; O'Neill *et al.*, 2008; Merino *et al.*, 2009).

- La familia de proteínas Bap

La familia de proteínas Bap (*Biofilm associated protein*) están emergiendo como elementos importantes en la etapa de adhesión intercelular en biofilms de diversas especies bacterianas (Tormo, Knecht, *et al.*, 2005; Lasa and Penadés, 2006). El primer miembro de este grupo de proteínas fue identificado por nuestro grupo, en un aislado de *S. aureus* procedente de una mastitis bovina y se denominó Bap (Cucarella *et al.*,

2001). La proteína Bap es una proteína de superficie de 2276 aminoácidos que presenta una región central formada por 13 repeticiones de 86 aminoácidos cada una, una secuencia señal de secreción extracelular en la región amino-terminal y un motivo LPXTG de anclaje al peptidoglicano en la región carboxilo-terminal. Nuestro grupo ha identificado la presencia de 4 hipotéticos motivos EF-hand de unión a calcio y ha demostrado que la funcionalidad de Bap está regulada por los niveles de calcio presentes en el medio (Arrizubieta *et al.*, 2004). Los aislados de *S. aureus* Bap-positivos son capaces de formar biofilm incluso en ausencia del exopolisacárido PIA/PNAG (Cucarella *et al.*, 2001; Cucarella *et al.*, 2004). En un estudio reciente, se ha visto que la proteína Bap es procesada y su región amino-terminal se autoensambla en agregados de tipo amiloide cuando el pH es ácido (<5) y la concentración de calcio inferior a 10 mM (Taglialegna *et al.*, 2016). Con respecto a la virulencia, Bap interactúa con el receptor celular Gp96, inhibiendo la invasión celular dando lugar a infecciones más persistentes y difíciles de erradicar (Cucarella *et al.*, 2001; Cucarella *et al.*, 2002; Cucarella *et al.*, 2004; Valle *et al.*, 2012).

- SasG

La proteína de superficie de *S. aureus* SasG promueve la formación de biofilms *in vitro* independientemente del polisacárido PIA/PNAG. Las cepas que expresan SasG forman fibrillas de densidad variable que enmascaran la unión de las proteínas MSCRAMMs a sus ligandos. La longitud de SasG parece ser crucial para enmascarar la unión de las proteínas a sus ligandos y la formación de biofilm. Las variantes de SasG con cinco o más repeticiones dentro del dominio B de la proteína forman biofilm y bloquean la unión de las proteínas MSCRAMMs a los ligandos, mientras que las variantes con menos repeticiones no lo hacen (Corrigan *et al.*, 2007). Además, se ha demostrado que el dominio A de SasG está involucrado en la mediación de la autoagregación intercelular por homo-oligomerización en el proceso de formación de biofilm (Kuroda *et al.*, 2008). Curiosamente, SasG requiere ser procesado proteolíticamente para inducir el desarrollo de biofilms (Corrigan *et al.*, 2007).

- FnBPs

Se han descrito varios aislados de *S. aureus* y *S. epidermidis* en los que las proteínas responsables de la formación de biofilm son las proteínas de unión a fibronectina A y B (FnBPA y FnBPB) (O'Neill *et al.*, 2007; O'Neill *et al.*, 2008; Vergara-

Irigaray *et al.*, 2009; Houston *et al.*, 2011). Uno de estos trabajos fue realizado en un aislado clínico, *S. aureus* 132, capaz de modular la composición de la matriz del biofilm dependiendo de las condiciones ambientales. En presencia de glucosa, la matriz del biofilm es de naturaleza proteica y requiere de las proteínas de unión a fibronectina (FnBP). Sin embargo, en condiciones de alta sal, la bacteria produce un biofilm de naturaleza polisacarídica dependiente de PIA/PNAG (Vergara-Irigaray *et al.*, 2009). Este trabajo, proporciona las primeras evidencias de que *S. aureus* es capaz de modular la composición de la matriz del biofilm en función de las condiciones ambientales. Las propiedades que cada tipo de biofilm aporta y las razones por las cuales cada tipo de matriz es mas conveniente en una determinada condición ambiental no han sido esclarecidas hasta el momento.

- Proteína A

Proteína A es ampliamente conocida por su capacidad para unirse a la fracción constante (Fc) de las IgG e inhibir la opsonofagocitosis (Forsgren and Sjöquist, 1966; Uhlén *et al.*, 1984). En un estudio previo se identificó que la sobreexpresión de proteína A promueve las interacciones bacteria-bacteria sin necesidad de estar anclada covalentemente al peptidoglicano, aunque el mecanismo exacto por el que Proteína A media la formación de biofilm es desconocido (Merino *et al.*, 2009).

- DNA extracelular

Otro componente importante de la matriz del biofilm de *S. aureus* junto al polisacárido PNAG y las proteínas, es el DNA extracelular (Whitchurch *et al.*, 2002). Durante el desarrollo del biofilm, la lisis celular y la liberación de DNA están críticamente reguladas por los genes *cidA* e *lrgAB* (Mann *et al.*, 2009). Se ha propuesto que una subpoblación del biofilm de *S. aureus* tiene un comportamiento al que se ha denominado de "*lisis altruista*", en el cual las células altruistas, a través de un proceso programado, lisan por el bien de la comunidad y la protección de las células supervivientes (Thomas *et al.*, 2008). El DNA extracelular (eDNA) funciona como una red electrostática para mantener unidas las células del biofilm. Además de ayudar a la estabilización de la matriz del biofilm, el eDNA juega un papel muy importante en los mecanismos de transferencia de genes entre bacterias y en el acondicionamiento de la respuesta inmune innata (Montanaro *et al.*, 2011).

En contraste con el PIA/PNAG y las proteínas que componen la matriz del biofilm, de los que se ha demostrado el impacto que tienen en las infecciones asociadas a biofilm en modelos animales usando mutantes, no resulta posible evaluar el papel que el eDNA juega durante el proceso de formación de biofilms *in vivo*, por lo que su papel en dicho proceso sigue siendo especulativo.

- Fibras amiloides

Las fibras amiloides son agregados polipeptídicos con una estructura tridimensional estable y sencilla, llamada lámina-β cruzada. Cada nanofibra está constituida por una cadena de monómeros idénticos que se autoensamblan como hebras en una lámina-β y que se yuxtaponen y se trenzan formando fibras solubles. La presencia de fibras amiloides parece ser una característica común de la matriz extracelular del biofilm en muy diversas bacterias. *S. aureus* produce fibras con propiedades amiloides como componentes de la matriz del biofilm. Estas fibras pueden estar constituidas por la proteína Bap, como se ha comentado anteriormente (Taglielegna et al., 2016), pero también por se han descrito fibras amiloides compuestas por unos pequeños péptidos conocidos como Phenol Soluble Modulins (PSM) (Schwartz et al., 2012). Los PSM pueden estar presentes como péptidos solubles o como estructuras amiloides polimerizadas. Cuando son solubles, los PSM adoptan una conformación de tipo α-hélice que es capaz de estimular la quimiotaxis de los neutrófilos, la formación de poros en la membrana de las células, y actúan como moléculas tensioactivas que favorecen la dispersión de las bacterias del biofilm. Bajo determinadas condiciones ambientales aún poco conocidas, los PSM adquieren una conformación de tipo lámina-β. En esta conformación, los péptidos se agregan y se ensamblan en estructuras fibrilares de tipo amiloide que proporcionan un soporte funcional a la matriz del biofilm (Schwartz et al., 2012; Schwartz et al., 2016).

2.2.3 Factores de dispersión del biofilm

A diferencia de la gran cantidad de información que se ha ido acumulando en relación con los componentes implicados en la formación del biofilm de *S. aureus*, se conoce muy poco sobre los mecanismos que subyacen en el proceso de dispersión de las bacterias del biofilm. En un estudio pionero, Ziebuhr et al. (Ziebuhr *et al.*, 1999) demostraron que la inserción/escisión del elemento de inserción IS256 en el operón *ica* juega un papel en la obtención de aislados biofilm negativos en *S. epidermidis*.

Además de los genes *ica*, otras dianas de inserción de IS256 que causan variantes biofilm negativos incluyen los genes *sarA* y *rsbU*, que son reguladores positivos de la expresión de *ica* (Conlon *et al.*, 2004; Valle *et al.*, 2007). La reversión a un fenotipo biofilm positivo de estos clones, requiere la escisión precisa de la secuencia de inserción IS256. En *S. aureus* también se ha demostrado variación de fase de la expresión de la proteína Bap, aunque los mecanismos que subyacen al proceso de variación de fase en Bap son desconocidos (Tormo *et al.*, 2007).

Un mecanismo de dispersión del biofilm basado en la degradación específica del exopolisacárido PIA/PNAG ha sido descrito en la bacteria *Aggregatibacter actinomycetemcomitans* (Kaplan *et al.*, 2003). Este grupo encontró que *A. actinomycetemcomitans* produce una enzima a la que denominaron dispersina B (DspB), que es capaz de hidrolizar los enlaces glicosídicos de poli-β-1,6-GlcNAc del PIA/PNAG, alterando la integridad de los biofilms de *S. aureus* y *S. epidermidis* (Kaplan, Ragunath, *et al.*, 2004; Itoh *et al.*, 2005; Rohde *et al.*, 2007; Izano *et al.*, 2008). Aunque sería razonable esperar que las especies que producen poli-β-1,6-N-acetilglucosaminoglicanos produjesen enzimas similares a la dispersina para permitir la dispersión de las bacterias al medio, no se han encontrado homólogos de DspB, en *S. aureus* ni en *S. epidermidis*. Por el contrario, el grupo de R. Patter identificó en un aislado clínico de *Staphylococcus lugduniensis*, un gen que codificaba una proteína con actividad glicosil hidrolasa similar a la dispersina (Frank and Patel, 2007). Finalmente, es interesante señalar que recientemente se ha publicado un artículo que muestra que la proteína PgaB del operon *pgaABCD* de *E. coli* (equivalente al operon *icaADBC*) tiene actividad hidrolasa del PNAG (Little *et al.*, 2018).

2.3. Regulación del desarrollo del biofilm en *S. aureus*

La formación de biofilms es un proceso costoso desde el punto de vista energético y se encuentra estrictamente controlado por una compleja red de reguladores que interactúan y se comunican entre ellos (Stoodley *et al.*, 2002; Ghigo, 2003). Muchos de los reguladores identificados hasta la fecha actúan directa o indirectamente a través de la regulación de la transcripción del operón *ica* o de otros elementos implicados en el proceso de formación del biofilm. En la siguiente sección, se discute el papel de varios reguladores (represores y activadores) del proceso de formación del biofilm de *S. aureus*.

2.3.1 Inductores del desarrollo de biofilms en *S. aureus*

- SarA

El regulador SarA juega un papel crucial en la regulación de los genes de virulencia en *S. aureus* (Cheung et al., 1992). En relación con la formación del biofilm de *S. aureus*, SarA se describió primero como un regulador negativo del desarrollo de biofilms (Pratten et al., 2001). Sin embargo, otros estudios han demostrado lo contrario y han destacado su papel esencial en el desarrollo de biofilms (Valle *et al.*, 2003; Beenken *et al.*, 2003; Beenken *et al.*, 2004; Cerca *et al.*, 2005; Tsang *et al.*, 2008). Nuestro grupo de investigación demostró que la mutación de *sarA* en cepas de *S. aureus* y *S. epidermidis* provoca una disminución de la expresión del operón *ica*, disminuyendo la producción de PIA/PNAG y perdiendo por completo la capacidad de formar biofilms (Valle *et al.*, 2003; Tormo, Martí, *et al.*, 2005). La capacidad de SarA para unirse al promotor *icaADBC* se ha demostrado, tanto en *S. aureus* como en *S. epidermidis* (Jefferson *et al.*, 2004; Tormo, Martí, *et al.*, 2005). Por otro lado, se ha demostrado que la incapacidad de los mutantes en *sarA* para reprimir la producción de nucleasas y proteasas extracelulares tiene efectos indirectos que contribuyen significativamente a la deficiencia en la formación de biofilms de los mutantes en *sarA* (Tsang *et al.*, 2008). SarA también controla positivamente la formación de biofilms dependiente de Bap por interacción directa con el promotor de *bap* (Trotonda *et al.*, 2005). Por otro lado, también se ha demostrado que el biofilm promovido por proteínas de unión a fibronectina depende de SarA. En este caso, SarA parece regular el proceso mediante el control de los niveles de actividad de proteasas extracelulares en lugar de mediante la regulación positiva de la transcripción de *fnbA* y *fnbB* (O'Neill *et al.*, 2008).

- Factor Sigma B

SigB es un factor de transcripción sigma alternativo de *S. aureus* que regula genes en condiciones de estrés (Chan *et al.*, 1998; Ferreira *et al.*, 2004; van Schaik and Abee, 2005). SigB es necesario en el proceso de formación de biofilm dependiente de proteínas, ya que la mutación de *sigB* incrementa los niveles de expresión de proteasas que degradarían las proteínas implicadas en el biofilm. Sin embargo, el papel de SigB en la regulación del biofilm dependiente de polisacárido PIA/PNAG es controvertida. Existen estudios en *S. epidermidis* que muestran que en ausencia de *sigB* la bacteria no es capaz de formar biofilm (Knobloch *et al.*, 2001; Handke *et al.*, 2007). De acuerdo con estos resultados, el grupo de W. Ziebuhr publicó un estudio en el que mostraron que la inactivación de *sigB* en *S. aureus* daba como resultado una disminución drástica de la

transcripción *ica* y una inhibición completa de la formación de biofilm (Rachid, Ohlsen, Wallner, *et al.*, 2000). Sin embargo, resultados de nuestro grupo muestran que la mutación de *sigB* en *S. aureus* apenas altera la transcripción del operón *icaADBC* y no afecta significativamente la producción de PIA/PNAG y la formación de biofilm (Valle *et al.*, 2003).

- Proteínas GGDEF

El mensajero secundario ácido diguanílico cíclico, c-di-GMP, es un regulador global de una gran variedad de procesos bacterianos tales como la motilidad, la formación de biofilm y la virulencia (García *et al.*, 2004; Ryjenkov *et al.*, 2005; Römling *et al.*, 2005; Tamayo *et al.*, 2007). La síntesis y degradación de c-di-GMP dependen, respectivamente, de las actividades enzimáticas de diguanilato ciclasa y fosfodiesterasa. El análisis de los genomas secuenciados ha mostrado que *S. aureus* contiene una proteína conservada con dominio GGDEF, GdpS, y una segunda proteína con un dominio GGDEF altamente modificado, GdpP, (Holland *et al.*, 2008). La mutación de *gdpS* reduce la expresión de *ica* y la producción de PIA/PNAG y afecta la formación de biofilm en condiciones estáticas y condiciones de flujo. Sin embargo, GdpS no parece ser capaz de sintetizar c-di-GMP , lo que sugiere que el control de GdpS en el desarrollo del biofilm ocurre por un mecanismo independiente de c-di-GMP (Holland *et al.*, 2008).

- Rbf

Se ha demostrado que el regulador de formación de biofilm (Rbf), miembro de la familia de reguladores transcripcionales AraC/XylS, activa el proceso de formación de biofilms de *S. aureus* en la etapa de agregación multicelular (Lim *et al.*, 2004; Luong *et al.*, 2009). Lim et al. demostraron que una mutación específica de rbf afectaba a la formación de biofilm inducido por glucosa o NaCl pero no por etanol. Curiosamente, la mutación de *rbf* no afecta a la transcripción de *ica* (Lim *et al.*, 2004).

- CidA

Los operones de *S. aureus cidABC* y *lrgAB* codifican un complejo sistema regulador que controla la muerte bacteriana. Se ha demostrado que la mutación de *cidA* disminuye la capacidad de *S. aureus* para formar biofilms. Este fenotipo se atribuye a la

menor liberación de ADN genómico extracelular que se encuentra en los mutantes *cidA* debido que se produce una reducción en la tasa de muerte celular (Rice *et al.*, 2007). La transcripción de *cidABC* y *lrgAB* depende de la presencia de los genes *alsSD* (Yang *et al.*, 2006). Se ha descrito que la mutación de *alsSD* en *S. aureus* da como resultado una disminución en la formación de biofilm que, de acuerdo con los datos anteriores, podría deberse a la represión de *cidA* (Cassat *et al.*, 2006).

- CcpA

El gen *ccpA* codifica la proteína de control de catabolitos A (CcpA) que regula la expresión génica en respuesta a la fuente de carbono. La eliminación de *ccpA* suprime la capacidad de *S. aureus* para formar biofilm en condiciones estáticas y de flujo continuo, aunque todavía permite la adhesión primaria a superficies de poliestireno. Se desconoce el mecanismo mediante el cual la disrupción de *ccpA* disminuye la adhesión intercelular pero se ha especulado que podría deberse, al menos en parte, a unos niveles reducidos de ADN genómico extracelular (Rice *et al.*, 2007).

- Sistemas de dos componentes

Los sistemas de dos componentes (TCS) representan el sistema sensorial mas habitual en bacterias que permite transmitir las condiciones ambientales al interior de la bacteria y adecuar la fisiología y metabolismo bacteriano a dichas condiciones. Consiste en un módulo de detección, representado por una proteína que en presencia de un estimulo se autofosforila en un residuo de histidina y luego transmite el grupo fosforilo al residuo aspártico de un regulador de respuesta. El regulador de respuesta fosforilado activa o reprime la expresión de genes y en algunos casos puede tener una actividad enzimática. El análisis del genoma completo reveló la presencia de 16 TCS en *S. aureus*. Entre los 16 sistemas de dos componentes de *S. aureus*, los sistemas WalKR, GraRS y SrrAB, se han descrito como activadores del proceso de formación del biofilm:

- **WalKR**

El TCS WalKR es específico de bacterias Gram-positivas con un bajo contenido en G+C y se ha demostrado que es esencial para la viabilidad celular (Winkler and Hoch, 2008). En *S. aureus* y en *Streptococcus mutans*, se ha demostrado que WalKR controla positivamente la formación de biofilms (Senadheera *et al.*, 2005; Dubrac *et al.*, 2007).

En *S. aureus*, se ha sugerido que la formación alterada de biofilms en cepas deficientes en WalKR está relacionada con la alteración en la expresión de autolisinas, ya que WalKR regula positivamente las dos principales autolisinas de *S. aureus*, AtlA y LytM (Dubrac *et al.*, 2007).

- **GraRS**

Existen datos contradictorios sobre el papel del TCS asociado a la resistencia a glucopéptidos (GraRS) en el proceso de formación de biofilms de *S. aureus*. Herbert et al. mostraron que la mutación de *graRS* en *S. aureus* inducía un fenotipo biofilm negativo (Herbert *et al.*, 2007). Este fenotipo se atribuyó a una pronunciada disminución en la expresión de los genes *ica*, *dlt* y *atlA*. Sin embargo, Shanks et al. mostraron que la mutación de *graRS* en *S. aureus* provocaba un aumento en la formación de biofilm (Shanks *et al.*, 2008). En este escenario, estudios adicionales van a ser necesarios para entender el papel que el sistema GraRS juega en el proceso de formación del biofilm.

- **SrrAB**

Como hemos mencionado antes, las condiciones anaeróbicas inducen la transcripción *ica* y la acumulación de PIA/PNAG (Cramton *et al.*, 2001). Se ha demostrado que el sistema de respuesta respiratoria estafilocócica (SrrAB) responde a los niveles de oxígeno ambiental y parece estar implicado en la conexión de los niveles de oxígeno con la producción de PIA/PNAG (Pragman *et al.*, 2004). En condiciones de bajo oxígeno, SrrAB aumenta significativamente la transcripción de *icaA* y la producción de PIA/PNAG mediante la unión directa de SrrA al promotor *icaADBC* (Ulrich *et al.*, 2007). Por el contrario, bajo condiciones aeróbicas se han publicado resultados contradictorios. Por ejemplo, Tu Quoc et al. mostraron que la alteración de *srrA*, a pesar de que induce la producción de PIA/PNAG, provoca una disminución en la capacidad de formación de biofilms (Tu Quoc *et al.*, 2007). Por el contrario, Ulrich et al. no observaron un aumento en la transcripción *ica* ni la producción de PIA/PNAG en mutantes *srrAB* bajo crecimiento aeróbico (Ulrich *et al.*, 2007). Por tanto, en estos momentos, lo único que se puede concluir es que el sistema SrrAB es un activador de la expresión de *ica* en condiciones anaeróbicas.

2.3.2 Represores del desarrollo de biofilms en *S. aureus*

- IcaR

El gen *icaR* se localiza delante del operón *ica,* pero en orientación opuesta, y codifica un represor transcripcional de la familia TetR de reguladores transcripcionales (Conlon *et al.*, 2002). IcaR se une a una región de 42 pb inmediatamente delante del gen *icaA* reprimiendo la transcripción del operón *icaADBC* (Jefferson *et al.*, 2003; Jefferson *et al.*, 2004). Por consiguiente, la deleción de *icaR* en *S. aureus* aumenta la producción de PIA/PNAG y la formación de biofilms. Una característica particular del gen icaR es que el mRNA contiene una larga región no traducida en el extremo 3' (3' UTR). Esta región 3' UTR proporciona un mecanismo de regulación post-transcripcional de la expresión de IcaR basado en la hibridación entre parte de la región 3´ UTR de icaR y la secuencia de unión al ribosoma (RBS) del mismo mRNA (Ruiz de Los Mozos *et al.*, 2013). La deleción o modificación del motivo complementario a la RBS (anti-RBS) de la 3´-UTR es suficiente para romper esta interacción y dar como resultado una mayor acumulación de IcaR y por lo tanto una mayor inhibición en la formación del biofilm. También se ha visto que antibióticos como la gentamicina activa la expresión de los genes *icaADBC* al interferir con el sitio de unión de IcaR (Jeng *et al.*, 2008).

- TcaR

TcaR, una proteína de la familia MarR, capaz de unirse al promotor de los genes *ica* (Jefferson *et al.*, 2004). Sin embargo, aunque la transcripción de los genes *ica* aumenta en ausencia de TcaR, ni la producción de PIA/PNAG ni la formación de biofilms se alteran significativamente (Jefferson *et al.*, 2004). Experimentos de epistasis realizados con *icaR* y *tcaR* revelaron que la doble disrupción de estos genes causaba un mayor aumento de la transcripción de *ica*, de la producción de PIA/PNAG y de la formación de biofilms que las mutaciones individuales (Jefferson *et al.*, 2004). Estos resultados sugieren que, aunque IcaR es un represor más potente y puede anular los efectos de eliminar TcaR, ambos reguladores afectan la producción de PIA/PNAG de *S. aureus* y la formación de biofilms a través de vías independientes (Jefferson *et al.*, 2004).

- MgrA

El gen *mgrA*, también llamado *ratA* o *norR*, fue identificado simultáneamente por tres laboratorios diferentes (Truong-Bolduc *et al.*, 2003; Ingavale *et al.*, 2003; Luong *et al.*, 2003). Pertenece a la familia de proteínas SarA (Cheung *et al.*, 2004) y codifica un regulador pleiotrópico que controla la autolisis (Ingavale *et al.*, 2003), la virulencia (Ingavale *et al.*, 2005) y la actividad de la bomba de expulsión en *S. aureus* (Truong-

Bolduc *et al.*, 2005). Recientemente, se ha demostrado que la inactivación de *mgrA* en *S. aureus* induce la formación de biofilm independientemente de *ica* favoreciendo la expresión de proteínas de superficie y la acumulación de DNA (Trotonda *et al.*, 2008). Recientemente, se ha demostrado que el aumento en la formación de biofilms de los mutante en MgrA se debe en parte a la regulación positiva de SasG, a través del sistema de dos-componentes ArlRS. Estos resultados confirmaron que ArlRS y MgrA constituyen una cascada reguladora, y que controlan la expresión de varios genes importantes para la virulencia y el biofilm, incluidas proteínas de superficie (Crosby *et al.*, 2016).

- SarZ

SarZ es un miembro de la familia SarA/MarR de reguladores transcripcionales, se identificó en primer lugar como un contribuyente de la producción de hemolisina y la virulencia en *S. aureus* (Kaito *et al.*, 2006). Posteriormente se observó que los mutantes *sarZ* tienen una mayor capacidad para formar biofilms mediado por proteínas. La capacidad de los mutantes *sarZ* para producir biofilms robustos se explica por la transcripción disminuida de *mgrA* y *agr* y la activación de la expresión de *sarA* (Tamber and Cheung, 2009).

- CodY

El regulador global CodY se descubrió por primera vez en *Bacillus subtilis*, donde se sabe que es una proteína de unión al GTP que controla la expresión de genes de fase estacionaria y el inicio de la esporulación (Ratnayake-Lecamwasam *et al.*, 2001). La mutación de *codY* en aislados clínicos de *S. aureus* dio como resultado un aumento en la transcripción de *ica* (Majerczyk *et al.*, 2008). Estos datos contrastan con los encontrados por Tu Quoc et al. que mostraron que la mutación *codY* en un aislado clínico de *S. aureus* da como resultado una disminución de la formación de biofilm y de la producción de PIA/PNAG (Tu Quoc *et al.*, 2007).

- Clp ATPasas

El genoma de *S. aureus* codifica para 5 proteínas de la familia Clp ATPasas, denominadas *clpB, clpC, clpL, clpX y clpY* (Frees *et al.*, 2004). ClpC había sido identificado previamente como un gen inducido en condiciones de biofilm (Becker *et al.*, 2001). Más tarde, se demostró que tanto ClpC como ClpX son necesarios para la formación de

biofilms en *S. aureus* (Frees *et al.*, 2004). Por el contrario, las bacterias deficientes en ClpP parecen ser más propensas a la formación de biofilms (Frees *et al.*, 2004). Curiosamente, se ha demostrado que los niveles de transcripción del operón ica desciende en un mutante ClpP (Michel *et al.*, 2006).

- Sistemas de dos componentes

Dos TCS, *agr* y *arlRS*, han sido descritos como represores del proceso de formación del biofilm.

- **AgrCA**

El sistema *agr* (gen regulador accesorio) es un sistema de dos-componentes y de *quorum sensing* que se activa durante la transición de la fase de crecimiento exponencial a la fase estacionaria, mediante un mecanismo autorregulador que implica la acumulación en el medio extracelular de un péptido (AIP) cuando la densidad celular supera un determinado umbral (Novick and Muir, 1999). El locus *agr* consiste en cuatro genes *agrBDCA* que están cotranscritos (RNAII) por el promotor P2 y una región genética que se transcribe en la dirección opuesta por el promotor P3 y codifica la molécula efectora del sistema *agr* (RNAIII) y el gen *hld* responsable de la producción de la toxina δ (Peng *et al.*, 1988; Janzon *et al.*, 1989). Durante el crecimiento celular, AgrD se sintetiza, se procesa proteolíticamente en AIP y se secreta a través de un mecanismo poco conocido que requiere la proteína de membrana AgrB (Zhang and Ji, 2004; Qiu *et al.*, 2005; Kavanaugh *et al.*, 2007). Cuando AIP alcanza una concentración crítica, interactúa con AgrC, el sensor del sistema de dos componentes, que activa a AgrA que es el regulador de respuesta induciendo la transcripción de ambos promotores P2 y P3 (Koenig *et al.*, 2004). La expresión del transcrito de P3, RNAIII, provoca la regulación positiva de los genes que codifican numerosas proteínas extracelulares y factores de virulencia y la regulación negativa de los genes de las proteínas de superficie (Dunman *et al.*, 2001). Numerosos estudios coinciden en señalar que *agr* juega un papel negativo en la formación de biofilms. Vuong et al. (Vuong *et al.*, 2000) vieron que la mutación *agr* en *S. aureus* inducía la formación de biofilm. Este efecto se atribuyó, al menos en parte, a las propiedades tensioactivas de la toxina δ. En esta misma línea, los estudios centrados en la funcionalidad de *agr* en aislados clínicos de *S. aureus*, han mostrado que los mutantes espontáneos de *agr,* circunstancia que ocurre con relativa frecuencia en condiciones de laboratorio, son mejores formadores de biofilm que las cepas *agr* positivas (Vuong *et al.*, 2000; Traber *et al.*, 2008). Otros autores han sugerido que *agr*

puede influir en la formación de biofilm a través de la regulación del proceso de dispersión (Yarwood *et al.*, 2004; Boles and Horswill, 2008). La activación del sistema *agr* en biofilm ya establecidos induce la dispersión a través de la activación de proteasas extracelulares. Estos hallazgos sugieren que el sistema de detección de quórum de *agr* puede mediar el cambio entre el estilo de vida sésil y planctónico, favoreciendo la dispersión de *S. aureus* y la colonización de nuevos sitios (Boles and Horswill, 2008).

- **ArlRS**

El sistema de dos componentes *arlRS* fue inicialmente relacionado con el proceso de autolisis de *S. aureus* (Fournier and Hooper, 2000). Sin embargo, la mutación de *arlRS* no tiene ningún efecto en la actividad de autolisis de cepas de *S. aureus* MRSA (Memmi *et al.*, 2012). Estudios previos de nuestro grupo mostraron que la mutación de *arlRS* afecta negativamente el desarrollo de biofilms de *S. aureus* en un medio mínimo químicamente definido HHWm (Toledo-Arana *et al.*, 2005). Estos resultados fueron consistentes con un estudio previo que mostraba que la mutación de *arlS* induce la formación de biofilms en superficies de poliestireno en medio rico TSB (Fournier and Hooper, 2000). Sin embargo, en este trabajo se relacionó la mayor capacidad para formar biofilm del mutante *arlS* con una mayor adherencia primaria debida a una mayor actividad autolítica (Fournier and Hooper, 2000). Por el contrario, nuestros resultados indicaban que la actividad autolisina no era responsable del aumento de biofilm observado en el mutante *arlRS* en HHWm y que el biofilm formado no requería de la presencia del polisacárido PIA/PNAG. En un trabajo posterior observamos que el mutante en *arlRS* produce mayores niveles de Proteína A y que era la acumulación de proteína A lo que podría explicar la mayor capacidad para formar biofilm de este mutante (Merino *et al.*, 2009; Villanueva *et al.*, 2018). En el caso de *S. epidermidis*, se ha visto que una cepa mutante en *arlRS* es incapaz de formar biofilm de naturaleza polisacarídica (PIA/PNAG). Esta reducción de los niveles PIA/PNAG además viene unido a la disminución de niveles de transcripción de varios genes implicados en la formación del biofilm, como *icaADBC*, *sigB* y *sarA* y al aumento de la expresión del represor *icaR* (Yang Wu *et al.*, 2012).

Como conclusión general en relación con la regulación del proceso de formación del biofilm de *S. aureus*, es importante señalar que a pesar de la gran cantidad de reguladores que han sido identificados, nuestra compresión sobre cómo se regula dicho proceso es todavía muy incipiente. Esto se debe principalmente a dos factores, por un

lado, existen muchos trabajos que muestran resultados contradictorios para un mismo regulador, y es difícil extraer conclusiones claras. La existencia de resultados contradictorios en estudios realizados en laboratorios independientes, se puede explicar en base a la utilización de cepas diferentes de *S. aureus* para realizar los estudios. En principio, la utilización de cepas genéticamente no relacionadas no debería ser un inconveniente, porque los resultados obtenidos deberían ser consistentes independientemente del fondo genético, pero la realidad es que esta es una situación bastante común en la genética de *S. aureus*.

Por otro lado, no se ha conseguido identificar relaciones epistáticas entre los distintos reguladores y parece que cada uno de los reguladores funciona con independencia del resto. Una posible explicación a esta situación es que exista redundancia en el proceso de regulación y que la expresión de estos factores sea diferente en las distintas cepas.

3. Estrategias para la prevención y el tratamiento de las infecciones de biofilms de *Staphylococcus aureus*

A lo largo de estos años se han establecido directrices nacionales e internacionales que recogen una serie de recomendaciones para intentar disminuir la incidencia de las infecciones asociadas a biofilms en la implantación de dispositivos médicos invasivos. Sin embargo, estas medidas no han sido suficientes para solventar el gran problema que estas infecciones suponen en el ámbito hospitalario. Por ello, existe un enorme interés por desarrollar estrategias que prevengan o faciliten el tratamiento de las infecciones estafilocócicas asociadas a dispositivos médicos. Estas estrategias se están dirigiendo a las diferentes etapas de formación del biofilm: i) adherencia primaria, ii) síntesis de la matriz y iii) dispersión. A continuación, voy a describir algunas estrategias de interrupción y tratamiento del biofilm que se han probado.

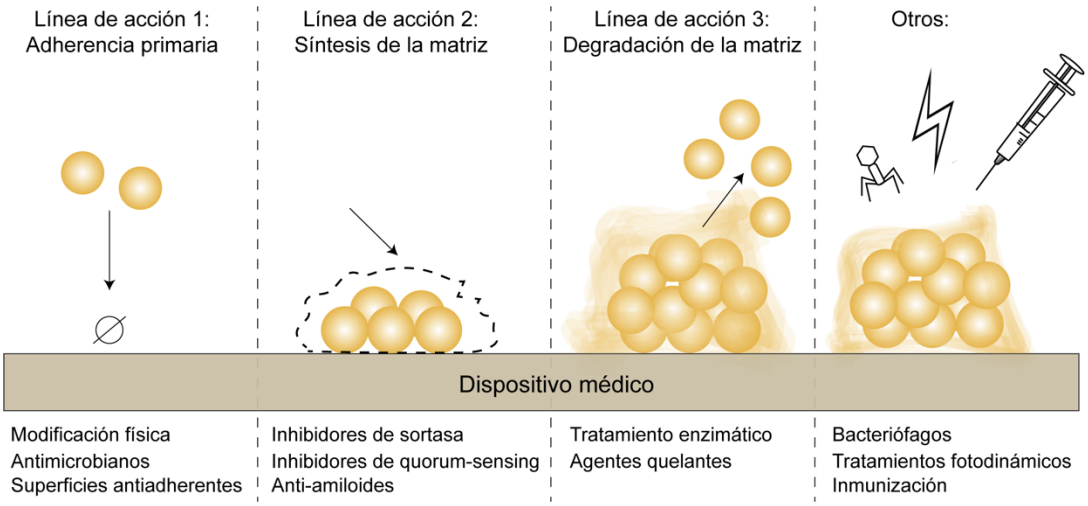


Figura 2. Estrategias para prevenir y tratar las infecciones asociadas a dispositivos médicos que afectan a las diferentes etapas del biofilm

3.1 Adherencia primaria: modificación de la superficie en implantes médicos

La primera línea de acción para combatir los biofilms microbianos consiste en evitar la adherencia de la bacteria a la superficie, para ello se están desarrollando superficies que eviten o dificulten la adherencia de las bacterias. El logro de materiales resistentes a la infección puede basarse en diferentes enfoques: (i) modificación física

de la superficie del biomaterial para proporcionar propiedades antiadherentes; (ii) modificación química de la superficie con propiedades antiadherentes; y (iii) recubrimiento de los materiales con sustancias antimicrobianas.

3.1.1 Modificación física de la superficie

En los últimos años se ha realizado un gran esfuerzo en entender cómo la topografía y los patrones físicos de las superficies influyen en la interacción con los microorganismos y alteran su adhesión y/o su proliferación sobre las superficies. Uno de los mecanismos para evitar la adhesión bacteriana sobre superficies consiste en crear superficies con topografías que reducen el área de contacto de las bacterias con la superficie mediante la impresión de un patrón periódico en el rango de 1 a 3 micras. Esta estrategia de modificar la superficie para que evite o dificulte la adherencia de las bacterias puede combinarse con el recubrimiento químico de las superficies con desinfectantes o antibióticos que eliminen aquellos microorganismos que logren adherirse irreversiblemente a la superficie.

3.1.2 Modificación de la superficie con sustancias antiadherentes

Las superficies hidrofílicas y las cargas negativas son menos propensas a la adhesión bacteriana que las superficies hidrofóbicas (Speziale *et al.*, 2008). La heparina, con sus fuertes propiedades hidrofílicas, se ha utilizado para evitar la adhesión bacteriana a superficies de catéteres y otros dispositivos médicos. Además de disminuir la hidrofobicidad formando una capa altamente hidratada entre las bacterias y la superficie, se ha demostrado que la heparina puede interferir con la adhesión de *S. epidermidis* inhibiendo específicamente la unión de adhesinas bacterianas a sus ligandos que se encuentran recubriendo la superficie del biomaterial (Arciola *et al.*, 1993; Arciola *et al.*, 1994; Arciola *et al.*, 1995; Arciola *et al.*, 1998; Arciola *et al.*, 2003; Bustanji *et al.*, 2003; Legeay *et al.*, 2006). Además de la heparina, se han utilizado recubrimientos de polímeros hidrofílicos de poli-etilenglicol (PEG) o poli-óxido de etileno (PEO). Una capa de estas cadenas poliméricas altamente hidratadas sobre una superficie inhibe la adhesión de proteínas del huésped y las bacterias (Neoh and Kang, 2011).

3.1.3 Modificación de la superficie con antimicrobianos

Una tercera aproximación se ha basado en conjugar agentes antimicrobianos, en particular antibióticos, a la superficie del biomaterial. Un inconveniente asociado a esta estrategia es el riesgo de que la presencia del antimicrobiano induzca la aparición de cepas resistentes (Campoccia *et al.*, 2010). Una sustancia antimicrobiana que se ha propuesto para recubrir la superficie de los implantes es el polisacárido catiónico quitosano. Ensayos con *S. aureus* y *P. aeruginosa* han mostrado que el titanio funcionalizado con quitosano y ácido láurico inhiben eficazmente la adhesión y el crecimiento de las bacterias (Zhao *et al.*, 2014). Los mismos resultados se han obtenido cuando se han tratado biofilms de *S. aureus* con nanopartículas de óxido de hierro recubiertas con quitosano (Shi *et al.*, 2016) o superficies de polimetacrilato (PMMA) cargados con quitosano en combinación con antibióticos. También se está trabajando para desarrollar estrategias que combinen la utilización de agentes antiadherentes y antimicrobianos. A modo de ejemplo, la película multicapa construida mediante el ensamblaje de heparina y quitosano, da lugar a un biomaterial con propiedades antiadhesivas y antibacterianas (Fu *et al.*, 2005). Otra alternativa son los péptidos catiónicos, que constituyen un componente importante de las defensas inmunes innatas y se ha demostrado que son capaces de eliminar una amplia variedad de bacterias Gram-negativas y Gram-positivas Kang:2012ef}. Se ha demostrado que los péptidos catiónicos inmovilizados sobre un polímero hidrofílico proporcionan un recubrimiento robusto con propiedades antiadhesivas y antimicrobianas, altamente eficaces para combatir la formación de biofilms (Hancock and Sahl, 2006; Bagheri *et al.*, 2012).

3.2 Síntesis de la matriz del biofilm

La segunda línea de acción para combatir los biofilms microbianos consiste en evitar que las bacterias que se han adherido a las superficies de los dispositivos médicos sinteticen los componentes de la matriz del biofilm y así impedir la formación de su matriz extracelular. De esta manera, es posible que las defensas del huésped sean capaces de resolver la infección y que los tratamientos antibióticos sean más eficaces.

3.2.1 Moléculas e inhibidores de sortasa

Debido al papel clave de la enzima sortasa (SrtA) en el anclaje a la pared celular de proteínas MSCRAMMs y de otras proteínas de superficie implicadas en las interacciones intercelulares del biofilm, se han realizado estudios para identificar moléculas que inhiban a esta enzima. Oh et al., identificaron una nueva clase de

inhibidores de la sortasa de *S. aureus*, los diarilacrilonitrilos (Oh *et al.*, 2010). Un derivado de esta molécula (Z)-3-(2,5-dimetoxifenil)-2-(4-metoxifenil) acrilonitrilo (DMMA) se ha visto que es eficaz en el tratamiento de infecciones óseas y articulares en un modelo de infección murino por *S. aureus* (Oh *et al.*, 2004).

3.2.2 Moléculas inhibidoras de quorum-sensing

Como hemos descrito anteriormente al describir el sistema de regulación *agr*, el quorum sensing (QS) es un mecanismo de comunicación entre bacterias que permite controlar procesos específicos dependiendo de la densidad bacteriana. El mecanismo de comunicación intercelular está basado en la producción de pequeñas moléculas llamadas autoinductores (AI), que se acumulan en el medio extracelular cuando la densidad de la población supera un determinado umbral. Se ha identificado una molécula denominada "furanona", producida por el alga *Delisea pulcra*, con una estructura similar a los AI bacterianos. Esta molécula se une a los mismos receptores y bloquea el sistema QS y la consiguiente formación de biofilm. En la actualidad se está intentando desarrollar inhibidores de la formación del biofilm basados en derivados de la furanona, ya que esta molécula es extremadamente tóxica (Hentzer *et al.*, 2002).

3.2.3 Moléculas anti-amiloides

La presencia de estructuras fibrilares con conformación amiloide parece ser una característica común de la matriz extracelular del biofilm en muy diversas bacterias. Se han descrito diversas proteínas que se ensamblan formando fibras amiloides como componentes de la matriz del biofilm en bacterias como CsgA en *E. coli*, TapA en *Bacillus,* FapA en *Pseudomonas* y Bap en *S. aureus* (Chapman et al., 2002; Romero et al., 2010; Dueholm et al., 2010). La mayoría de las proteínas amiloidogénicas tienen una secuencia (llamada núcleo), que es la unidad básica amiloide para formar las fibrillas. Estas secuencias se consideran regiones de auto-reconocimiento, a través del las cuales las proteínas interactúan formando fibras. Se ha visto que moléculas como los polifenoles (Epigalocatequin galato), péptidos cortos y anticuerpos que interfieren en el núcleo de determinadas proteínas amiloides, evitan la oligomerización de las fibras amiloides y la formación de biofilm (Serra *et al.*, 2016). Varios autores han propuesto la posibilidad de utilizar compuestos antiamiloides para inhibir la formación de la matriz del biofilm (Romero *et al.*, 2013; Taglialegna *et al.*, 2016).

3.3 Degradación de la matriz del biofilm

Una vez que el biofilm se ha formado sobre un dispositivo médico, otra posible línea de acción incluye la utilización agentes, compuestos o enzimas que desestabilicen el biofilm o que disuelvan los polímeros de la matriz del biofilm.

3.3.1 Tratamiento enzimático del biofilm

La lisostafina causa la lisis de *S. aureus* al romper los puentes de pentaglicina del peptidoglicano de la pared celular y se ha demostrado que tiene propiedades anti-biofilm *in vitro* (Dajcs *et al.*, 2000; Julie A Wu *et al.*, 2003; Kokai-Kun *et al.*, 2009). Se cree que la lisostafina no tiene un efecto enzimático directo sobre las células eucariotas y tiene poca toxicidad (Kokai-Kun *et al.*, 2009).

Otra enzima prometedora para el tratamiento de biofilms de *S. aureus* asociados a dispositivos médicos es la dispersina B (DspB). DspB es una enzima glucósido hidrolasa producida por *Aggregatibacter actinomycetemcomitans*, un patógeno periodontal humano (Donelli *et al.*, 2007). Se ha demostrado que DspB tiene la capacidad de hidrolizar y degradar el polisacárido PIA/PNAG producido por varias especies de estafilococos durante la formación del biofilm, incluso a concentraciones muy bajas (Kaplan, Velliyagounder, *et al.*, 2004; Kaplan, 2009). La efectividad de la DspB contra los biofilms de *S. epidermidis* y *S. aureus* independientes de PIA/PNAG es menos clara, pero podría usarse en combinación con agentes dirigidos a otros constituyentes del biofilm estafilocócico. La utilización de proteasas extracelulares (proteinasa K, tripsina y pancreatina) puede representar otra opción profiláctica o terapéutica en el proceso de dispersión de biofilms dependientes de proteínas (Chaignon *et al.*, 2007; Joo-Hyeon Park *et al.*, 2012; Kumar Shukla and Rao, 2013).

Como hemos visto anteriormente, el eDNA es otro de los componentes esenciales de la matriz de biofilm de *S. aureus* (Kaplan, 2009). La enzima DNAsa I es una endonucleasa que rompe enlaces fosfodiéster en diferentes regiones ubicadas en el interior de una cadena polinucleotídica. Esta enzima tiene un papel en la inhibición y desestabilización del biofilm de *S. aureus* (Eckhart *et al.*, 2007). Se cree que la inhibición de la formación de biofilm por DNasa I ocurre por la degradación de los ácidos nucleicos asociados a la superficie celular que funcionan como adhesinas superficiales (Qin *et al.*, 2007; Kaplan, 2009) o por la degradación directa del eDNA (Seidl *et al.*, 2008). Con respecto a la disgregación del biofilm, los estudios han demostrado que la DNAsa I es más efectiva en las primeras etapas de formación del biofilms que en los biofilms

maduros (Qin *et al.*, 2007). En este sentido, es interesante señalar que aerosoles conteniendo DNase I has sido utilizados para el tratamiento de infecciones crónicas causadas por biofilms en pacientes de fibrosis quística (van der Giessen *et al.*, 2007).

3.3.2 Agentes quelantes y dispersantes del biofilm

Los agentes quelantes pueden desestabilizar la arquitectura de la matriz de biofilm y facilitar su disolución. El citrato de sodio es uno de los quelantes que tiene un efecto inhibidor sobre el biofilm de *S. aureus* (Shanks *et al.*, 2006). En esta misma línea, estudios *in vitro* han mostrado la eficacia del EDTA en la erradicación del biofilm (Kite *et al.*, 2004; Percival *et al.*, 2005). La combinación de EDTA disódico y agentes antimicrobianos como la tigeciclina o la gentamicina se ha visto que es efectiva para reducir la formación de biofilms y la eliminación bacteriana tanto en especies de estafilococos como en *P. aeruginosa* (Bookstaver *et al.*, 2009). La combinación Minocycline-EDTA (M-EDTA) mostró un efecto sinérgico en la prevención de la colonización y de la formación de biofilms (Raad *et al.*, 2007). Esta combinación fue efectiva tanto en biofilms recién formados (*in vitro*) como en biofilms maduros (*ex vivo*) (Raad *et al.*, 2003). El papel del hierro también ha sido fruto de estudio en el proceso de formación de biofilms estafilocócicos. Varios estudios han demostrado que el hierro regula positivamente la formación del biofilm estafilocócicos (Lin *et al.*, 2012). Por consiguiente, un agente quelante de hierro, la apotransferrina, inhibe el biofilm de *S. aureus* y *S. epidermidis* (Ardehali *et al.*, 2002).

La N-acetil-L-cisteína es capaz de disgregar el biofilm producido por *S. aureus* en discos de polietileno y titanio, sugiriendo un posible papel en el tratamiento de infecciones protésicas causadas por *S. aureus* (Drago *et al.*, 2013). También se ha visto que un compuesto de origen vegetal, 1, 2, 3, 4, 6-Penta-O-galloyl- β-D-glucopiranosa (PGG), que tiene un bajo perfil de citotoxicidad, es capaz de inhibir la formación de biofilm en *S. aureus* (Lin *et al.*, 2011). Aunque se ha demostrado que los agentes quelantes son efectivos en algunos estudios, se necesita investigar más para ayudar a determinar su seguridad y eficacia.

3.4 Otros mecanismos anti-biofilm

3.4.1 Bacteriófagos

Los bacteriófagos son virus que actúan como parásitos obligados capaces de invadir células bacterianas, inyectar su material genómico y hacerse cargo del sistema metabólico del huésped. La idea de utilizar bacteriófagos para el tratamiento de infecciones causadas por biofilms de *Staphylococcus* ha sido propuesta por varios autores (Del Pozo *et al.*, 2007, Wittebole *et al.*, 2014). Sin embargo, los estudios publicados sobre el efecto de la terapia con fagos en modelos animales son contradictorios. En un modelo de osteomielitis, la utilización de fagos junto con antibióticos produjo resultados satisfactorios (Fernández-Hidalgo *et al.*, 2010). En cambio, en un modelo de infección de tejidos blandos en rata, se ha visto que es necesario disgregar previamente el biofilm antes del tratamiento con fagos para la eliminación completa del mismo, lo que indica que el acceso del fago a las células del biofilm se encuentra restringida por la matriz extracelular (Yilmaz *et al.*, 2013; Seth *et al.*, 2013). Una de las limitaciones que debe superar la utilización de fagos en terapia es su elevada especificidad. La mayoría de los fagos son cepa específicos, lo que significa que solo son capaces de infectar a un tipo de cepa y no al resto. Otra alternativa que se ha evaluado recurrentemente es la utilización de enzimas líticas de los fagos, como endolisisnas e hidrolasas del peptidoglicano asociadas al virion, para combatir los biofilms (Hughes *et al.*, 1998; Sutherland *et al.*, 2004; Sillankorva *et al.*, 2004; Gutiérrez *et al.*, 2018). En este sentido, las enzimas líticas del fago muestran una actividad antimicrobiana de espectro reducido y un reducido desarrollo de resistencias. Además, el uso terapéutico de enzimas líticas de fagos en modelos de infección animal de *S. aureus* está dando resultados prometedores, mostrando buena eficacia sin efectos secundarios aparentes. No obstante, los ensayos clínicos en humanos todavía están en progreso, y los datos aún no están disponibles.

3.4.2 Tratamientos fotodinámicos del biofilm

Un enfoque interesante y novedoso para el tratamiento de infecciones por biofilms estafilocócicos es el uso de la terapia fotodinámica (TFD), un proceso donde los microorganismos se tratan con un fármaco fotosensibilizador y posteriormente se iluminan con una longitud de onda adecuada (Jori, 2006). Este proceso genera especies de oxígeno reactivo y radicales libres que ejercen un efecto bactericida sobre biofilms adheridos a dispositivos médicos, sin afectar a las células eucariotas. Sharma et al.

describieron la inactivación significativa de *S. epidermidis* y una cepa de MRSA expuesta a azul de toluidina (TBO) y tratamiento con láser (Sharma *et al.*, 2008). Un estudio reciente demostró la actividad de la clorina altamente pura, utilizada como fotosensibilizador de segunda generación, en la erradicación del biofilm de *S. aureus* en un modelo de infección murino (Jong-Hwan Park *et al.*, 2012).

3.4.3 Inmunización

Aunque la mayoría de los enfoques de inmunoterapia estafilocócica se han dirigido a inducir protección frente a procesos de infección aguda, la utilización de inmunización o vacunas para prevenir la formación de biofilms de *S. aureus* es una estrategia que merece la pena ser considerada. Como hemos visto anteriormente, la matriz del biofilm de *S. aureus* está compuesta por polisacáridos, proteínas y eDNA en distintas cantidades según las cepas y las condiciones ambientales. La mayoría de los estudios para generar una respuesta inmune frente al biofilm de *S. aureus* se han dirigido utilizando como antígeno el polisacárido PIA/PNAG. El grupo de J. Pier demostró que la inmunización activa o pasiva con PIA/PNAG protege contra la infección por *S. aureus* en un modelo de infección renal (McKenney *et al.*, 1999). Sin embargo, trabajos posteriores del mismo grupo han indicado que la efectividad de la respuesta inmune frente a este antígeno es variable (Maira-Litràn *et al.*, 2002). Por otro lado, el hecho de que no todos los aislados de *S. aureus* sintetizan PIA/PNAG, al menos en las condiciones de laboratorio, sugiere la necesidad de tener que incorporar otros antígenos para poder generar una respuesta inmune eficaz frente a infecciones mediadas por biofilms de *S. aureus*.

Tabla 5. Compuestos químicos, enzimas y agentes utilizados en el tratamiento del biofilm de *S. aureus*

Adherencia primaria	
Heparina	(Arciola *et al.*, 2003; Bustanji *et al.*, 2003)
Poli-etilenglicol (PEG)	(Neoh and Kang, 2011)
Poli-óxido de etileno (POG)	(Neoh and Kang, 2011)
Quitosano	(Zhao *et al.*, 2014; Shi *et al.*, 2016)
Péptidos antimicrobianos	(Hancock and Sahl, 2006; Bagheri *et al.*, 2012)
Síntesis de la matriz del biofilm	
(Z)-3-(2,5-dimetoxifenil)-2-(4-metoxifenil) acrilonitrilo (DMMA)	(Oh *et al.*, 2004)
Furanona	(Hentzer *et al.*, 2002)
Péptido RIP	(Gov *et al.*, 2001)
Epigalocatequin galato (EGCG)	(Taglialegna *et al.*, 2016)
Degradación de la matriz	
Lisostafina	(Julie A Wu *et al.*, 2003)
Dispersina B	(Kaplan, 2009)
Proteinasa K	(Joo-Hyeon Park *et al.*, 2012; Kumar Shukla and Rao, 2013)
Tripsina	(Chaignon *et al.*, 2007)
Pancreatina	(Chaignon *et al.*, 2007)
DNasa I	(Eckhart *et al.*, 2007)
Citrato de sodio	(Shanks *et al.*, 2006)
EDTA	(Kite *et al.*, 2004; Percival *et al.*, 2005; Bookstaver *et al.*, 2009)
Minocycline-EDTA	(Raad *et al.*, 2007)
Apotransferrina,	(Ardehali *et al.*, 2002)
N-acetil-L-cisteína	(Drago *et al.*, 2013)
2, 3, 4, 6-Penta-O-galloyl-□-D-glucopiranosa (PGG)	(Lin *et al.*, 2011)
Otros	
Bacteriófagos	(Yilmaz *et al.*, 2013; Seth *et al.*, 2013; Gutiérrez *et al.*, 2016)
Bacteriolisinas	(Sutherland *et al.*, 2004; Sillankorva *et al.*, 2004; Yilmaz *et al.*, 2013)
Azul de toluidina (TBO)	(Sharma *et al.*, 2008)
Clorina	(Jong-Hwan Park *et al.*, 2012)

REFERENCIAS

Abraham, N.M., and Jefferson, K.K. (2012) Staphylococcus aureus clumping factor B mediates biofilm formation in the absence of calcium. *Microbiology* **158**: 1504–1512.

Allegranzi, B., Borg, M., Brenner, P., Bruce, M., Callery, S., Damani, N., *et al.* (2007) *IFIC Basic Concepts of Infection Control*. 2nd ed., Washington.

Arciola, C.R., An, Y.H., Campoccia, D., Donati, M.E., and Montanaro, L. (2005) Etiology of implant orthopedic infections: a survey on 1027 clinical isolates. *The International Journal of Artificial Organs* **28**: 1091–1100.

Arciola, C.R., Baldassarri, L., and Montanaro, L. (2001) Presence of icaA and icaD genes and slime production in a collection of staphylococcal strains from catheter-associated infections. *J Clin Microbiol* **39**: 2151–2156.

Arciola, C.R., Bustanji, Y., Conti, M., Campoccia, D., Baldassarri, L., Samorì, B., and Montanaro, L. (2003) Staphylococcus epidermidis-fibronectin binding and its inhibition by heparin. *Biomaterials* **24**: 3013–3019.

Arciola, C.R., Caramazza, R., and Pizzoferrato, A. (1994) In vitro adhesion of Staphylococcus epidermidis on heparin-surface-modified intraocular lenses. *J Cataract Refract Surg* **20**: 158–161.

Arciola, C.R., Cenni, E., Caramazza, R., and Pizzoferrato, A. (1995) Seven surgical silicones retain Staphylococcus aureus differently in vitro. *Biomaterials* **16**: 681–684.

Arciola, C.R., Montanaro, L., Caramazza, R., Sassoli, V., and Cavedagna, D. (1998) Inhibition of bacterial adherence to a high-water-content polymer by a water-soluble, nonsteroidal, anti-inflammatory drug. *J Biomed Mater Res* **42**: 1–5.

Arciola, C.R., Radin, L., Alvergna, P., Cenni, E., and Pizzoferrato, A. (1993) Heparin surface treatment of poly(methylmethacrylate) alters adhesion of a Staphylococcus aureus strain: utility of bacterial fatty acid analysis. *Biomaterials* **14**: 1161–1164.

Ardehali, R., Shi, L., Janatova, J., Mohammad, S.F., and Burns, G.L. (2002) The effect of apo-transferrin on bacterial adhesion to biomaterials. *Artif Organs* **26**: 512–520.

Arrizubieta, M.J., Toledo-Arana, A., Amorena, B., Penadés, J.R., and Lasa, I. (2004) Calcium inhibits bap-dependent multicellular behavior in Staphylococcus aureus. *Journal of Bacteriology* **186**: 7490–7498.

Bagheri, M., Beyermann, M., and Dathe, M. (2012) Mode of action of cationic antimicrobial peptides defines the tethering position and the efficacy of biocidal surfaces. *Bioconjug Chem* **23**: 66–74.

Baldassarri, A.M., Masson, S., Marchettini, P., Lazzerini, A., and Boicelli, A. (1996) Reinnervation and recovery of muscle energetics after nerve section and surgical repair. *J Neurol* **243**: 421–422.

Barrio, B., Vangroenweghe, F., Dosogne, H., and Burvenich, C. (2000) Decreased neutrophil bactericidal activity during phagocytosis of a slime-producing Staphylococcus aureus strain. *Vet Res* **31**: 603–609.

Becker, P., Hufnagle, W., Peters, G., and Herrmann, M. (2001) Detection of differential gene expression in biofilm-forming versus planktonic populations of Staphylococcus aureus using micro-representational-difference analysis. *Appl Environ Microbiol* **67**: 2958–2965.

Beenken, K.E., Blevins, J.S., and Smeltzer, M.S. (2003) Mutation of sarA in Staphylococcus aureus limits biofilm formation. *Infection and Immunity* **71**: 4206–4211.

Beenken, K.E., Dunman, P.M., McAleese, F., Macapagal, D., Murphy, E., Projan, S.J., *et al.* (2004) Global gene expression in Staphylococcus aureus biofilms. *Journal of Bacteriology* **186**: 4665–4684.

Biswas, R., Voggu, L., Simon, U.K., Hentschel, P., Thumm, G., and Götz, F. (2006) Activity of the major staphylococcal autolysin Atl. *FEMS Microbiol Lett* **259**: 260–268.

Boles, B., and Horswill, A. (2008) Agr-mediated dispersal of Staphylococcus aureus biofilms. *PLoS Pathog* – http://dx.plos.org/10.1371/journal.ppat.1000052.

Bookstaver, P.B., Williamson, J.C., Tucker, B.K., Raad, I.I., and Sherertz, R.J. (2009) Activity of novel antibiotic lock solutions in a model against isolates of catheter-related bloodstream infections. *Ann Pharmacother* **43**: 210–219.

Brady, R.A., Leid, J.G., Calhoun, J.H., Costerton, J.W., and Shirtliff, M.E. (2008) Osteomyelitis and the role of biofilms in chronic infection. *FEMS Immunol Med Microbiol* **52**: 13–22.

Bustanji, Y., Arciola, C.R., Conti, M., Mandello, E., Montanaro, L., and Samorì, B. (2003) Dynamics of the interaction between a fibronectin molecule and a living bacterium under mechanical force. *Proc Natl Acad Sci USA* **100**: 13292–13297.

Cafiso, V., Bertuccio, T., Santagati, M., Campanile, F., Amicosante, G., Perilli, M.G., *et al.* (2004) Presence of the ica operon in clinical isolates of Staphylococcus epidermidis and its role in biofilm production. *Clin Microbiol Infect* **10**: 1081–1088.

Campoccia, D., Montanaro, L., Speziale, P., and Arciola, C.R. (2010) Antibiotic-loaded biomaterials and the risks for the spread of antibiotic resistance following their prophylactic and therapeutic clinical use. *Biomaterials* **31**: 6363–6377.

Cassat, J., Dunman, P.M., Murphy, E., Projan, S.J., Beenken, K.E., Palm, K.J., *et al.* (2006) Transcriptional profiling of a Staphylococcus aureus clinical isolate and its isogenic agr and sarA mutants reveals global differences in comparison to the laboratory strain RN6390. *Microbiology (Reading, Engl)* **152**: 3075–3090.

Cerca, N., Jefferson, K.K., Maira-Litràn, T., Pier, D.B., Kelly-Quintos, C., Goldmann, D.A., *et al.* (2007) Molecular basis for preferential protective efficacy of antibodies

directed to the poorly acetylated form of staphylococcal poly-N-acetyl-beta-(1-6)-glucosamine. *Infection and Immunity* **75**: 3406–3413.

Cerca, N., Pier, G.B., Vilanova, M., Oliveira, R., and Azeredo, J. (2005) Quantitative analysis of adhesion and biofilm formation on hydrophilic and hydrophobic surfaces of clinical isolates of Staphylococcus epidermidis. *Research in Microbiologoy* **156**: 506–514.

Chaignon, P., Sadovskaya, I., Ragunah, C., Ramasubbu, N., Kaplan, J.B., and Jabbouri, S. (2007) Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. *Appl Microbiol Biotechnol* **75**: 125–132.

Chapman, M.R., Robinson, L.S., Pinkner, J.S., Roth, R., Heuser, J., Hammar, M., *et al.* (2002) Role of Escherichia coli curli operons in directing amyloid fiber formation. *Science* **295**: 851–855.

Chan, P.F., Foster, S.J., Ingham, E., and Clements, M.O. (1998) The Staphylococcus aureus alternative sigma factor sigmaB controls the environmental stress response but not starvation survival or pathogenicity in a mouse abscess model. *Journal of Bacteriology* **180**: 6082–6089.

Chastre, J., and Fagon, J.-Y. (2002) Ventilator-associated pneumonia. *Am J Respir Crit Care Med* **165**: 867–903.

Chavakis, T., Wiechmann, K., Preissner, K.T., and Herrmann, M. (2005) Staphylococcus aureus interactions with the endothelium: the role of bacterial "secretable expanded repertoire adhesive molecules" (SERAM) in disturbing host defense systems. *Thromb Haemost* **94**: 278–285.

Cheung, A.L., Bayer, A.S., Zhang, G., Gresham, H., and Xiong, Y.-Q. (2004) Regulation of virulence determinants in vitro and in vivo in Staphylococcus aureus. *FEMS Immunol Med Microbiol* **40**: 1–9.

Chhatwal, G.S., and Blobel, H. (1987) Heterogeneity of fibronectin reactivity among streptococci as revealed by binding of fibronectin fragments. *Comp Immunol Microbiol Infect Dis* **10**: 99–108.

Christensen, G.D., Barker, L.P., Mawhinney, T.P., Baddour, L.M., and Simpson, W.A. (1990) Identification of an antigenic marker of slime production for Staphylococcus epidermidis. *Infection and Immunity* **58**: 2906–2911.

Clarke, S.R., and Foster, S.J. (2006) Surface adhesins of Staphylococcus aureus. *Adv Microb Physiol* **51**: 187–224.

Conlon, K.M., Humphreys, H., and O'Gara, J.P. (2002) icaR encodes a transcriptional repressor involved in environmental regulation of ica operon expression and biofilm formation in Staphylococcus epidermidis. *Journal of Bacteriology* **184**: 4400–4408.

Conlon, K.M., Humphreys, H., and O'Gara, J.P. (2004) Inactivations of rsbU and sarA by IS256 represent novel mechanisms of biofilm phenotypic variation in Staphylococcus epidermidis. *Journal of Bacteriology* **186**: 6208–6219.

Corrigan, R.M., Rigby, D., Handley, P., and Foster, T.J. (2007) The role of Staphylococcus aureus surface protein SasG in adherence and biofilm formation. *Microbiology (Reading, Engl)* **153**: 2435–2446.

Corvec, S., Portillo, M.E., Pasticci, B.M., Borens, O., and Trampuz, A. (2012) Epidemiology and new developments in the diagnosis of prosthetic joint infection. *The International Journal of Artificial Organs* **35**: 923–934.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., and Lappin-Scott, H.M. (1995) Microbial biofilms. *Annu Rev Microbiol* **49**: 711–745.

Costerton, J.W., Stewart, P.S., and Greenberg, E.P. (1999) Bacterial biofilms: a common cause of persistent infections. *Science* **284**: 1318–1322.

Cramton, S.E., Gerke, C., Schnell, N.F., Nichols, W.W., and Gotz, F. (1999) The intercellular adhesion (ica) locus is present in Staphylococcus aureus and is required for biofilm formation. *Infection and Immunity* **67**: 5427–5433.

Cramton, S.E., Ulrich, M., Gotz, F., and Döring, G. (2001) Anaerobic conditions induce expression of polysaccharide intercellular adhesin in Staphylococcus aureus and Staphylococcus epidermidis. *Infection and Immunity* **69**: 4079–4085.

Crosby, H.A., Schlievert, P.M., Merriman, J.A., King, J.M., Salgado-Pabón, W., and Horswill, A.R. (2016) The Staphylococcus aureus Global Regulator MgrA Modulates Clumping and Virulence by Controlling Surface Protein Expression. *PLoS Pathog* **12**: e1005604.

Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I., and Penadés, J.R. (2001) Bap, a Staphylococcus aureus surface protein involved in biofilm formation. *Journal of Bacteriology* **183**: 2888–2896.

Cucarella, C., Tormo, M.A., Knecht, E., Amorena, B., Lasa, I., Foster, T.J., and Penadés, J.R. (2002) Expression of the biofilm-associated protein interferes with host protein receptors of Staphylococcus aureus and alters the infective process. *Infection and Immunity* **70**: 3180–3186.

Cucarella, C., Tormo, M.A., Úbeda, C., Trotonda, M.P., Monzón, M., Peris, C., *et al.* (2004) Role of biofilm-associated protein bap in the pathogenesis of bovine Staphylococcus aureus. *Infection and Immunity* **72**: 2177–2185.

Dajcs, J.J., Hume, E.B., Moreau, J.M., Caballero, A.R., Cannon, B.M., and O'Callaghan, R.J. (2000) Lysostaphin treatment of methicillin-resistant Staphylococcus aureus keratitis in the rabbit. *Investigative ophthalmology & visual science* **41**: 1432–1437.

Del Pozo, J.L., Alonso, M., Arciola, C.R., Gonzalez, R., Leiva, J., Lasa, I., and Penades, J. (2007) Biotechnological war against biofilms. Could phages mean the end of device-related infections? *The International Journal of Artificial Organs* **30**: 805–812.

Delmi, M., Vaudaux, P., Lew, D.P., and Vasey, H. (1994) Role of fibronectin in staphylococcal adhesion to metallic surfaces used as models of orthopaedic devices. *Journal of Orthopaedic Research* **12**: 432–438.

Donelli, G., Francolini, I., Romoli, D., Guaglianone, E., Piozzi, A., Ragunath, C., and Kaplan, J.B. (2007) Synergistic activity of dispersin B and cefamandole nafate in inhibition of staphylococcal biofilm growth on polyurethanes. *Antimicrobial Agents and Chemotherapy* **51**: 2733–2740.

Donlan, R.M. (2001) Biofilms and device-associated infections. *Emerging Infect Dis* **7**: 277–281.

Donlan, R.M., and Costerton, J.W. (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* **15**: 167–193.

Drago, L., De Vecchi, E., Mattina, R., and Romanò, C.L. (2013) Activity of N-acetyl-L-cysteine against biofilm of Staphylococcus aureus and Pseudomonas aeruginosa on orthopedic prosthetic materials. *The International Journal of Artificial Organs* **36**: 39–46.

Dubrac, S., Boneca, I.G., Poupel, O., and Msadek, T. (2007) New Insights into the WalK/WalR (YycG/YycF) Essential Signal Transduction Pathway Reveal a Major Role in Controlling Cell Wall Metabolism and Biofilm Formation in *Staphylococcus aureus*. *Journal of Bacteriology* **189**: 8257–8269.

Dueholm, M.S., Petersen, S.V., Sønderkær, M., Larsen, P., Christiansen, G., Hein, K.L., *et al.* (2010) Functional amyloid in Pseudomonas. *Molecular Microbiology* **77**: 1009–1020.

Dunman, P.M., Murphy, E., Haney, S., Palacios, D., Tucker-Kellogg, G., Wu, S., *et al.* (2001) Transcription profiling-based identification of Staphylococcus aureus genes regulated by the agr and/or sarA loci. *Journal of Bacteriology* **183**: 7341–7353.

Eckhart, L., Fischer, H., Barken, K.B., Tolker-Nielsen, T., and Tschachler, E. (2007) DNase1L2 suppresses biofilm formation by Pseudomonas aeruginosa and Staphylococcus aureus. *Br J Dermatol* **156**: 1342–1345.

Eiff, von, C., Heilmann, C., and Peters, G. (1999) New aspects in the molecular basis of polymer-associated infections due to staphylococci. *Eur J Clin Microbiol Infect Dis* **18**: 843–846.

Eiff, von, C., Jansen, B., Kohnen, W., and Becker, K. (2005) Infections associated with medical devices: pathogenesis, management and prophylaxis. *Drugs* **65**: 179–214.

Fernández-Hidalgo, N., Gavaldà, J., Almirante, B., Martín, M.-T., Onrubia, P.L., Gomis, X., and Pahissa, A. (2010) Evaluation of linezolid, vancomycin, gentamicin and ciprofloxacin in a rabbit model of antibiotic-lock technique for Staphylococcus aureus catheter-related infection. *Journal of Antimicrobial Chemotherapy* **65**: 525–530.

Ferreira, A., Gray, M., Wiedmann, M., and Boor, K.J. (2004) Comparative genomic analysis of the sigB operon in Listeria monocytogenes and in other Gram-positive bacteria. *Current microbiology* **48**: 39–46.

Fitzpatrick, F., Humphreys, H., and O'Gara, J.P. (2005) Evidence for icaADBC-independent biofilm development mechanism in methicillin-resistant Staphylococcus aureus clinical isolates. *J Clin Microbiol* **43**: 1973–1976.

Flock, J.I., Fröman, G., Jönsson, K., Guss, B., Signäs, C., Nilsson, B., *et al.* (1987) Cloning and expression of the gene for a fibronectin-binding protein from Staphylococcus aureus. *EMBO J* **6**: 2351–2357.

Fluckiger, U., Ulrich, M., Steinhuber, A., Döring, G., Mack, D., Landmann, R., *et al.* (2005) Biofilm formation, icaADBC transcription, and polysaccharide intercellular adhesin synthesis by staphylococci in a device-related infection model. *Infection and Immunity* **73**: 1811–1819.

Forsgren, A., and Sjöquist, J. (1966) "Protein A" from S. aureus. I. Pseudo-immune reaction with human gamma-globulin. *J Immunol* **97**: 822–827.

Foster, T.J. (2005) Immune evasion by staphylococci. *Nat Rev Microbiol* **3**: 948–958.

Foster, T.J., and Höök, M. (1998) Surface protein adhesins of Staphylococcus aureus. *Trends in Microbiology* **6**: 484–488.

Fournier, B., and Hooper, D.C. (2000) A new two-component regulatory system involved in adhesion, autolysis, and extracellular proteolytic activity of *Staphylococcus aureus*. *Journal of Bacteriology* **182**: 3955–3964.

Fowler, V.G., Fey, P.D., Reller, L.B., Chamis, A.L., Corey, G.R., and Rupp, M.E. (2001) The intercellular adhesin locus ica is present in clinical isolates of Staphylococcus aureus from bacteremic patients with infected and uninfected prosthetic joints. *Med Microbiol Immunol* **189**: 127–131.

Francois, P., Tu Quoc, P.H., Bisognano, C., Kelley, W.L., Lew, D.P., Schrenzel, J., *et al.* (2003) Lack of biofilm contribution to bacterial colonisation in an experimental model of foreign body infection by Staphylococcus aureus and Staphylococcus epidermidis. *FEMS Immunol Med Microbiol* **35**: 135–140.

Frank, K.L., and Patel, R. (2007) Poly-N-Acetylglucosamine Is Not a Major Component of the Extracellular Matrix in Biofilms Formed by icaADBC-Positive Staphylococcus lugdunensis Isolates. *Infection and Immunity* **75**: 4728–4742.

Frees, D., Chastanet, A., Qazi, S., Sørensen, K., Hill, P., Msadek, T., and Ingmer, H. (2004) Clp ATPases are required for stress tolerance, intracellular replication and biofilm formation in Staphylococcus aureus. *Molecular Microbiology* **54**: 1445–1462.

Fu, J., Ji, J., Yuan, W., and Shen, J. (2005) Construction of anti-adhesive and antibacterial multilayer films via layer-by-layer assembly of heparin and chitosan. *Biomaterials* **26**: 6684–6692.

García, B., Latasa, C., Solano, C., García-del Portillo, F., Gamazo, C., and Lasa, I. (2004) Role of the GGDEF protein family in Salmonella cellulose biosynthesis and biofilm formation. *Molecular Microbiology* **54**: 264–277.

Gerke, C., Kraft, A., Süssmuth, R., Schweitzer, O., and Gotz, F. (1998) Characterization of the N-acetylglucosaminyltransferase activity involved in the biosynthesis of the Staphylococcus epidermidis polysaccharide intercellular adhesin. *J Biol Chem* **273**: 18586–18593.

Ghigo, J.-M. (2003) Are there biofilm-specific physiological pathways beyond a reasonable doubt? *Research in Microbiologoy* **154**: 1–8.

Gordon, R.J., and Lowy, F.D. (2008) Pathogenesis of methicillin-resistant Staphylococcus aureus infection. *Clin Infect Dis* **46 Suppl 5**: S350–9.

Gov, Y., Bitler, A., Dell'Acqua, G., Torres, J.V., and Balaban, N. (2001) RNAIII inhibiting peptide (RIP), a global inhibitor of Staphylococcus aureus pathogenesis: structure and function analysis. *Peptides* **22**: 1609–1620.

Götz, F. (2002) Staphylococcus and biofilms. *Molecular Microbiology* **43**: 1367–1378.

Guggenbichler, J.P., Assadian, O., Boeswald, M., and Kramer, A. (2011) Incidence and clinical implication of nosocomial infections associated with implantable biomaterials - catheters, ventilator-associated pneumonia, urinary tract infections. *GMS Krankenhhyg Interdiszip* **6**: Doc18.

Gutiérrez, D., Fernández, L., Rodríguez, A., and García, P. (2018) Are Phage Lytic Proteins the Secret Weapon To Kill Staphylococcus aureus? *MBio* **9**: e01923–17–17.

Gutiérrez, D., Hidalgo-Cantabrana, C., Rodríguez, A., García, P., and Ruas-Madiedo, P. (2016) Monitoring in Real Time the Formation and Removal of Biofilms from Clinical Related Pathogens Using an Impedance-Based Technology. *PLoS ONE* **11**: e0163966.

Hancock, R.E.W., and Sahl, H.-G. (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotechnol* **24**: 1551–1557.

Handke, L.D., Slater, S.R., Conlon, K.M., O'Donnell, S.T., Olson, M.E., Bryant, K.A., *et al.* (2007) SigmaB and SarA independently regulate polysaccharide intercellular adhesin production in Staphylococcus epidermidis. *Can J Microbiol* **53**: 82–91.

Heilmann, C., Hussain, M., Peters, G., and Gotz, F. (1997) Evidence for autolysin-mediated primary attachment of Staphylococcus epidermidis to a polystyrene surface. *Molecular Microbiology* **24**: 1013–1024.

Hentzer, M., Riedel, K., Rasmussen, T.B., Heydorn, A., Andersen, J.B., Parsek, M.R., *et al.* (2002) Inhibition of quorum sensing in Pseudomonas aeruginosa biofilm bacteria by a halogenated furanone compound. *Microbiology (Reading, Engl)* **148**: 87–102.

Herbert, S., Bera, A., Nerz, C., Kraus, D., Peschel, A., Goerke, C., *et al.* (2007) Molecular Basis of Resistance to Muramidase and Cationic Antimicrobial Peptide Activity of Lysozyme in *Staphylococci*. *PLoS Pathog* **3**: e102.

Holland, L.M., O'Donnell, S.T., Ryjenkov, D.A., Gomelsky, L., Slater, S.R., Fey, P.D., *et al.* (2008) A staphylococcal GGDEF domain protein regulates biofilm formation independently of cyclic dimeric GMP. *Journal of Bacteriology* **190**: 5178–5189.

Houston, P., Rowe, S.E., Pozzi, C., Waters, E.M., and O'Gara, J.P. (2011) Essential role for the major autolysin in the fibronectin-binding protein-mediated Staphylococcus aureus biofilm phenotype. *Infection and Immunity* **79**: 1153–1165.

Hughes, K.A., Sutherland, I.W., and Jones, M.V. (1998) Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. *Microbiology (Reading, Engl)* **144 (Pt 11)**: 3039–3047.

Hugonnet, S., Sax, H., Eggimann, P., Chevrolet, J.-C., and Pittet, D. (2004) Nosocomial bloodstream infection and clinical sepsis. *Emerging Infect Dis* **10**: 76–81.

Høiby, N., Bjarnsholt, T., Givskov, M., Molin, S., and Ciofu, O. (2010) Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial Agents* **35**: 322–332.

Ingavale, S., van Wamel, W., Luong, T.T., Lee, C.Y., and Cheung, A.L. (2005) Rat/MgrA, a regulator of autolysis, is a regulator of virulence genes in Staphylococcus aureus. *Infection and Immunity* **73**: 1423–1431.

Ingavale, S.S., Van Wamel, W., and Cheung, A.L. (2003) Characterization of RAT, an autolysis regulator in Staphylococcus aureus. *Molecular Microbiology* **48**: 1451–1466.

Itoh, Y., Wang, X., Hinnebusch, B.J., Preston, J.F., and Romeo, T. (2005) Depolymerization of beta-1,6-N-acetyl-D-glucosamine disrupts the integrity of diverse bacterial biofilms. *Journal of Bacteriology* **187**: 382–387.

Izano, E.A., Amarante, M.A., Kher, W.B., and Kaplan, J.B. (2008) Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in Staphylococcus aureus and Staphylococcus epidermidis biofilms. *Appl Environ Microbiol* **74**: 470–476.

Janzon, L., Löfdahl, S., and Arvidson, S. (1989) Identification and nucleotide sequence of the delta-lysin gene, hld, adjacent to the accessory gene regulator (agr) of Staphylococcus aureus. *Mol Gen Genet* **219**: 480–485.

Jefferson, K.K., Cramton, S.E., Götz, F., and Pier, G.B. (2003) Identification of a 5-nucleotide sequence that controls expression of the ica locus in Staphylococcus aureus and characterization of the DNA-binding properties of IcaR. *Molecular Microbiology* **48**: 889–899.

Jefferson, K.K., Pier, D.B., Goldmann, D.A., and Pier, G.B. (2004) The Teicoplanin-Associated Locus Regulator (TcaR) and the Intercellular Adhesin Locus Regulator (IcaR) Are Transcriptional Inhibitors of the ica Locus in Staphylococcus aureus. *Journal of Bacteriology* **186**: 2449–2456.

Jeng, W.-Y., Ko, T.-P., Liu, C.-I., Guo, R.-T., Liu, C.-L., Shr, H.-L., and Wang, A.H.-J. (2008) Crystal structure of IcaR, a repressor of the TetR family implicated in biofilm formation in Staphylococcus epidermidis. *Nucleic Acids Res* **36**: 1567–1577.

Johnson, M., Cockayne, A., and Morrissey, J.A. (2008) Iron-regulated biofilm formation in Staphylococcus aureus Newman requires ica and the secreted protein Emp. *Infection and Immunity* **76**: 1756–1765.

Jones, R.N. (2008) Key considerations in the treatment of complicated staphylococcal infections. *Clin Microbiol Infect* **14 Suppl 2**: 3–9.

Jori, G. (2006) Photodynamic therapy of microbial infections: state of the art and perspectives. *J Environ Pathol Toxicol Oncol* **25**: 505–519.

Jönsson, K., Signäs, C., Müller, H.P., and Lindberg, M. (1991) Two different genes encode fibronectin binding proteins in Staphylococcus aureus. The complete nucleotide sequence and characterization of the second gene. *Eur J Biochem* **202**: 1041–1048.

Kaito, C., Morishita, D., Matsumoto, Y., Kurokawa, K., and Sekimizu, K. (2006) Novel DNA binding protein SarZ contributes to virulence in Staphylococcus aureus. *Molecular Microbiology* **62**: 1601–1617.

Kaplan, J.B. (2009) Therapeutic potential of biofilm-dispersing enzymes. *The International Journal of Artificial Organs* **32**: 545–554.

Kaplan, J.B., Ragunath, C., Ramasubbu, N., and Fine, D.H. (2003) Detachment of Actinobacillus actinomycetemcomitans biofilm cells by an endogenous beta-hexosaminidase activity. *Journal of Bacteriology* **185**: 4693–4698.

Kaplan, J.B., Ragunath, C., Velliyagounder, K., Fine, D.H., and Ramasubbu, N. (2004) Enzymatic detachment of Staphylococcus epidermidis biofilms. *Antimicrobial Agents and Chemotherapy* **48**: 2633–2636.

Kaplan, J.B., Velliyagounder, K., Ragunath, C., Rohde, H., Mack, D., Knobloch, J.K.-M., and Ramasubbu, N. (2004) Genes involved in the synthesis and degradation of matrix polysaccharide in Actinobacillus actinomycetemcomitans and Actinobacillus pleuropneumoniae biofilms. *Journal of Bacteriology* **186**: 8213–8220.

Kathju, S., Nistico, L., Hall-Stoodley, L., Post, J.C., Ehrlich, G.D., and Stoodley, P. (2009) Chronic surgical site infection due to suture-associated polymicrobial biofilm. *Surg Infect (Larchmt)* **10**: 457–461.

Kavanaugh, J.S., Thoendel, M., and Horswill, A.R. (2007) A role for type I signal peptidase in Staphylococcus aureus quorum sensing. *Molecular Microbiology* **65**: 780–798.

Kennedy, A.D., Otto, M., Braughton, K.R., Whitney, A.R., Chen, L., Mathema, B., *et al.* (2008) Epidemic community-associated methicillin-resistant Staphylococcus aureus: recent clonal expansion and diversification. *Proc Natl Acad Sci USA* **105**: 1327–1332.

Kite, P., Eastwood, K., Sugden, S., and Percival, S.L. (2004) Use of in vivo-generated biofilms from hemodialysis catheters to test the efficacy of a novel antimicrobial catheter lock for biofilm eradication in vitro. *J Clin Microbiol* **42**: 3073–3076.

Kluytmans, J., van Belkum, A., and Verbrugh, H. (1997) Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks. *Clin Microbiol Rev* **10**: 505–520.

Knobloch, J.K.M., Bartscht, K., Sabottke, A., Rohde, H., Feucht, H.H., and Mack, D. (2001) Biofilm Formation by Staphylococcus epidermidis Depends on Functional RsbU, an Activator of the sigB Operon: Differential Activation Mechanisms Due to Ethanol and Salt Stress. *Journal of Bacteriology* **183**: 2624–2633.

Koenig, R.L., Ray, J.L., Maleki, S.J., Smeltzer, M.S., and Hurlburt, B.K. (2004) Staphylococcus aureus AgrA binding to the RNAIII-agr regulatory region. *Journal of Bacteriology* **186**: 7549–7555.

Kogan, G., Sadovskaya, I., Chaignon, P., Chokr, A., and Jabbouri, S. (2006) Biofilms of clinical strains of Staphylococcus that do not contain polysaccharide intercellular adhesin. *FEMS Microbiol Lett* **255**: 11–16.

Kokai-Kun, J.F., Chanturiya, T., and Mond, J.J. (2009) Lysostaphin eradicates established Staphylococcus aureus biofilms in jugular vein catheterized mice. *Journal of Antimicrobial Chemotherapy* **64**: 94–100.

Kostakioti, M., Hadjifrangiskou, M., and Hultgren, S.J. (2013) Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. *Cold Spring Harb Perspect Med* **3**: a010306–a010306.

Kraus, D., and Peschel, A. (2008) Staphylococcus aureus evasion of innate antimicrobial defense. *Future Microbiol* **3**: 437–451.

Kristian, S.A., Golda, T., Ferracin, F., Cramton, S.E., Neumeister, B., Peschel, A., *et al.* (2004) The ability of biofilm formation does not influence virulence of Staphylococcus aureus and host response in a mouse tissue cage infection model. *Microb Pathog* **36**: 237–245.

Kumar Shukla, S., and Rao, T.S. (2013) Dispersal of Bap-mediated Staphylococcus aureus biofilm by proteinase K. *J Antibiot* **66**: 55–60.

Kuroda, M., Ito, R., Tanaka, Y., Yao, M., Matoba, K., Saito, S., *et al.* (2008) Staphylococcus aureus surface protein SasG contributes to intercellular autoaggregation of Staphylococcus aureus. *Biochem Biophys Res Commun* **377**: 1102–1106.

Kurtz, S., Ong, K., Lau, E., Mowat, F., and Halpern, M. (2007) Projections of primary and revision hip and knee arthroplasty in the United States from 2005 to 2030. *J Bone Joint Surg Am* **89**: 780–785.

Lasa, I., and Penadés, J.R. (2006) Bap: a family of surface proteins involved in biofilm formation. *Research in Microbiologoy* **157**: 99–107.

Legeay, G., Poncin-Epaillard, F., and Arciola, C.R. (2006) New surfaces with hydrophilic/hydrophobic characteristics in relation to (no)bioadhesion. *The International Journal of Artificial Organs* **29**: 453–461.

Lim, Y., Jana, M., Luong, T.T., and Lee, C.Y. (2004) Control of glucose- and NaCl-induced biofilm formation by rbf in Staphylococcus aureus. *Journal of Bacteriology* **186**: 722–729.

Lin, M.-H., Chang, F.-R., Hua, M.-Y., Wu, Y.-C., and Liu, S.-T. (2011) Inhibitory effects of 1,2,3,4,6-penta-O-galloyl-beta-D-glucopyranose on biofilm formation by Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy* **55**: 1021–1027.

Lin, M.-H., Shu, J.-C., Huang, H.-Y., and Cheng, Y.-C. (2012) Involvement of iron in biofilm formation by Staphylococcus aureus. *PLoS ONE* **7**: e34388.

Little, D.J., Pfoh, R., Le Mauff, F., Bamford, N.C., Notte, C., Baker, P., *et al.* (2018) PgaB orthologues contain a glycoside hydrolase domain that cleaves deacetylated poly-β(1,6)-N-acetylglucosamine and can disrupt bacterial biofilms. *PLoS Pathog* **14**: e1006998.

Lopes, J.D., Reis, dos, M., and Brentani, R.R. (1985) Presence of laminin receptors in Staphylococcus aureus. *Science* **229**: 275–277.

Luong, T.T., Lei, M.G., and Lee, C.Y. (2009) Staphylococcus aureus Rbf activates biofilm formation in vitro and promotes virulence in a murine foreign body infection model. *Infection and Immunity* **77**: 335–340.

Luong, T.T., Newell, S.W., and Lee, C.Y. (2003) Mgr, a novel global regulator in Staphylococcus aureus. *Journal of Bacteriology* **185**: 3703–3710.

Mack, D., Fischer, W., Krokotsch, A., Leopold, K., Hartmann, R., Egge, H., and Laufs, R. (1996) The intercellular adhesin involved in biofilm accumulation of Staphylococcus epidermidis is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. *Journal of Bacteriology* **178**: 175–183.

Mack, D., Nedelmann, M., Krokotsch, A., Schwarzkopf, A., Heesemann, J., and Laufs, R. (1994) Characterization of transposon mutants of biofilm-producing Staphylococcus epidermidis impaired in the accumulative phase of biofilm production: genetic identification of a hexosamine-containing polysaccharide intercellular adhesin. *Infection and Immunity* **62**: 3244–3253.

Maira-Litràn, T., Kropec, A., Abeygunawardana, C., Joyce, J., Mark, G., Goldmann, D.A., and Pier, G.B. (2002) Immunochemical properties of the staphylococcal poly-N-acetylglucosamine surface polysaccharide. *Infection and Immunity* **70**: 4433–4440.

Majerczyk, C.D., Sadykov, M.R., Luong, T.T., Lee, C., Somerville, G.A., and Sonenshein, A.L. (2008) Staphylococcus aureus CodY negatively regulates virulence gene expression. *Journal of Bacteriology* **190**: 2257–2265.

Mann, E.E., Rice, K.C., Boles, B.R., Endres, J.L., Ranjit, D., Chandramohan, L., *et al.* (2009) Modulation of eDNA release and degradation affects Staphylococcus aureus biofilm maturation. *PLoS ONE* **4**: e5822.

Martín-López, J.V., Pérez-Roth, E., Claverie-Martín, F., Díez Gil, O., Batista, N., Morales, M., and Méndez-Alvarez, S. (2002) Detection of Staphylococcus aureus Clinical Isolates Harboring the ica Gene Cluster Needed for Biofilm Establishment. *J Clin Microbiol* **40**: 1569–1570.

McDevitt, D., Francois, P., Vaudaux, P., and Foster, T.J. (1994) Molecular characterization of the clumping factor (fibrinogen receptor) of Staphylococcus aureus. *Molecular Microbiology* **11**: 237–248.

McKenney, D., Hübner, J., Muller, E., Wang, Y., Goldmann, D.A., and Pier, G.B. (1998) The ica locus of Staphylococcus epidermidis encodes production of the capsular polysaccharide/adhesin. *Infection and Immunity* **66**: 4711–4720.

McKenney, D., Pouliot, K.L., Wang, Y., Murthy, V., Ulrich, M., Döring, G., *et al.* (1999) Broadly protective vaccine for Staphylococcus aureus based on an in vivo-expressed antigen. *Science* **284**: 1523–1527.

Memmi, G., Nair, D.R., and Cheung, A. (2012) Role of ArlRS in autolysis in methicillin-sensitive and methicillin-resistant Staphylococcus aureus strains. *Journal of Bacteriology* **194**: 759–767.

Merino, N., Toledo-Arana, A., Vergara-Irigaray, M., Valle, J., Solano, C., Calvo, E., *et al.* (2009) Protein A-mediated multicellular behavior in Staphylococcus aureus. *Journal of Bacteriology* **191**: 832–843.

Mermel, L.A. (2000) Prevention of intravascular catheter-related infections. *Ann Intern Med* **132**: 391–402.

Michel, A., Agerer, F., Hauck, C.R., Herrmann, M., Ullrich, J., Hacker, J., and Ohlsen, K. (2006) Global regulatory impact of ClpP protease of Staphylococcus aureus on regulons involved in virulence, oxidative stress response, autolysis, and DNA repair. *Journal of Bacteriology* **188**: 5783–5796.

Montanaro, L., Poggi, A., Visai, L., Ravaioli, S., Campoccia, D., Speziale, P., and Arciola, C.R. (2011) Extracellular DNA in biofilms. *The International Journal of Artificial Organs* **34**: 824–831.

Murdoch, D.R., Roberts, S.A., Fowler, V.G., Shah, M.A., Taylor, S.L., Morris, A.J., and Corey, G.R. (2001) Infection of orthopedic prostheses after Staphylococcus aureus bacteremia. *Clin Infect Dis* **32**: 647–649.

Neoh, K.G., and Kang, E.T. (2011) Combating bacterial colonization on metals via polymer coatings: relevance to marine and medical applications. *ACS Appl Mater Interfaces* **3**: 2808–2819.

Novick, R.P., and Muir, T.W. (1999) Virulence gene regulation by peptides in staphylococci and other Gram-positive bacteria. *Current Opinion in Microbiology* **2**: 40–45.

O'Neill, E., Pozzi, C., Houston, P., Humphreys, H., Robinson, D.A., Loughman, A., *et al.* (2008) A novel Staphylococcus aureus biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. *Journal of Bacteriology* **190**: 3835–3850.

O'Neill, E., Pozzi, C., Houston, P., Smyth, D., Humphreys, H., Robinson, D.A., and O'Gara, J.P. (2007) Association between methicillin susceptibility and biofilm regulation in Staphylococcus aureus isolates from device-related infections. *J Clin Microbiol* **45**: 1379–1388.

OECD (2016) Hip and knee replacement. In *Health at a Glance: Europe 2016*. OECD Publishing, pp. 172–173.

Oh, K.-B., Kim, S.-H., Lee, J., Cho, W.-J., Lee, T., and Kim, S. (2004) Discovery of diarylacrylonitriles as a novel series of small molecule sortase A inhibitors. *J Med Chem* **47**: 2418–2421.

Oh, K.-B., Nam, K.-W., Ahn, H., Shin, J., Kim, S., and Mar, W. (2010) Therapeutic effect of (Z)-3-(2,5-dimethoxyphenyl)-2-(4-methoxyphenyl) acrylonitrile (DMMA) against Staphylococcus aureus infection in a murine model. *Biochem Biophys Res Commun* **396**: 440–444.

Oshida, T., Sugai, M., Komatsuzawa, H., Hong, Y.M., Suginaka, H., and Tomasz, A. (1995) A Staphylococcus aureus autolysin that has an N-acetylmuramoyl-L-alanine amidase domain and an endo-beta-N-acetylglucosaminidase domain: cloning, sequence analysis, and characterization. *Proc Natl Acad Sci USA* **92**: 285–289.

O'Toole, G., Kaplan, H.B., and Kolter, R. (2000) Biofilm formation as microbial development. *Annu Rev Microbiol* **54**: 49–79.

Otto, M. (2013) Staphylococcal infections: mechanisms of biofilm maturation and detachment as critical determinants of pathogenicity. *Annu Rev Med* **64**: 175–188.

Park, Jong-Hwan, Ahn, M.-Y., Kim, Y.-C., Kim, S.-A., Moon, Y.-H., Ahn, S.-G., and Yoon, J.-H. (2012) In vitro and in vivo antimicrobial effect of photodynamic therapy using a highly pure chlorin e6 against Staphylococcus aureus Xen29. *Biol Pharm Bull* **35**: 509–514.

Park, Joo-Hyeon, Lee, J.-H., Cho, M.H., Herzberg, M., and Lee, J. (2012) Acceleration of protease effect on Staphylococcus aureus biofilm dispersal. *FEMS Microbiol Lett* **335**: 31–38.

Patti, J.M., Allen, B.L., McGavin, M.J., and Höök, M. (1994) MSCRAMM-Mediated Adherence of Microorganisms to Host Tissues. *Annu Rev Microbiol* **48**: 585–617.

Peng, H.L., Novick, R.P., Kreiswirth, B., Kornblum, J., and Schlievert, P. (1988) Cloning, characterization, and sequencing of an accessory gene regulator (agr) in Staphylococcus aureus. *Journal of Bacteriology* **170**: 4365–4372.

Percival, S.L., Kite, P., Eastwood, K., Murga, R., Carr, J., Arduino, M.J., and Donlan, R.M. (2005) Tetrasodium EDTA as a novel central venous catheter lock solution against biofilm. *Infect Control Hosp Epidemiol* **26**: 515–519.

Pragman, A.A., Yarwood, J.M., Tripp, T.J., and Schlievert, P.M. (2004) Characterization of virulence factor regulation by SrrAB, a two-component system in *Staphylococcus aureus*. *Journal of Bacteriology* **186**: 2430–2438.

Qin, Z., Ou, Y., Yang, L., Zhu, Y., Tolker-Nielsen, T., Molin, S., and Qu, D. (2007) Role of autolysin-mediated DNA release in biofilm formation of Staphylococcus epidermidis. *Microbiology (Reading, Engl)* **153**: 2083–2092.

Qiu, R., Pei, W., Zhang, L., Lin, J., and Ji, G. (2005) Identification of the putative staphylococcal AgrB catalytic residues involving the proteolytic cleavage of AgrD to generate autoinducing peptide. *J Biol Chem* **280**: 16695–16704.

Raad, I., Chatzinikolaou, I., Chaiban, G., Hanna, H., Hachem, R., Dvorak, T., *et al.* (2003) In vitro and ex vivo activities of minocycline and EDTA against microorganisms embedded in biofilm on catheter surfaces. *Antimicrobial Agents and Chemotherapy* **47**: 3580–3585.

Raad, I., Hanna, H., Dvorak, T., Chaiban, G., and Hachem, R. (2007) Optimal antimicrobial catheter lock solution, using different combinations of minocycline, EDTA, and 25-percent ethanol, rapidly eradicates organisms embedded in biofilm. *Antimicrobial Agents and Chemotherapy* **51**: 78–83.

Rachid, S., Ohlsen, K., Wallner, U., Hacker, J., Hecker, M., and Ziebuhr, W. (2000) Alternative transcription factor sigma(B) is involved in regulation of biofilm expression in a Staphylococcus aureus mucosal isolate. *Journal of Bacteriology* **182**: 6824–6826.

Rachid, S., Ohlsen, K., Witte, W., Hacker, J., and Ziebuhr, W. (2000) Effect of subinhibitory antibiotic concentrations on polysaccharide intercellular adhesin expression in biofilm-forming Staphylococcus epidermidis. *Antimicrobial Agents and Chemotherapy* **44**: 3357–3363.

Ratnayake-Lecamwasam, M., Serror, P., Wong, K.W., and Sonenshein, A.L. (2001) Bacillus subtilis CodY represses early-stationary-phase genes by sensing GTP levels. *Genes Dev* **15**: 1093–1103.

Rice, K.C., Mann, E.E., Endres, J.L., Weiss, E.C., Cassat, J.E., Smeltzer, M.S., and Bayles, K.W. (2007) The cidA murein hydrolase regulator contributes to DNA release and biofilm development in Staphylococcus aureus. *Proc Natl Acad Sci USA* **104**: 8113–8118.

Rohde, H., Burandt, E.C., Siemssen, N., Frommelt, L., Burdelski, C., Wurster, S., *et al.* (2007) Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of Staphylococcus epidermidis and Staphylococcus aureus isolated from prosthetic hip and knee joint infections. *Biomaterials* **28**: 1711–1720.

Rohde, H., Burdelski, C., Bartscht, K., Hussain, M., Buck, F., Horstkotte, M.A., *et al.* (2005) Induction of Staphylococcus epidermidis biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. *Molecular Microbiology* **55**: 1883–1895.

Romero, D., Aguilar, C., Losick, R., and Kolter, R. (2010) Amyloid fibers provide structural integrity to Bacillus subtilis biofilms. *Proc Natl Acad Sci USA* **107**: 2230–2234.

Romero, D., Sanabria-Valentín, E., Vlamakis, H., and Kolter, R. (2013) Biofilm inhibitors that target amyloid proteins. *Chem Biol* **20**: 102–110.

Römling, U., Gomelsky, M., and Galperin, M.Y. (2005) C-di-GMP: the dawning of a novel bacterial signalling system. *Molecular Microbiology* **57**: 629–639.

Ruiz de Los Mozos, I., Vergara-Irigaray, M., Segura, V., Villanueva, M., Bitarte, N., Saramago, M., *et al.* (2013) Base Pairing Interaction between 5"- and 3-"UTRs Controls icaR mRNA Translation in Staphylococcus aureus. *PLoS Genet* **9**: e1004001.

Ryjenkov, D.A., Tarutina, M., Moskvin, O.V., and Gomelsky, M. (2005) Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: insights into biochemistry of the GGDEF protein domain. *Journal of Bacteriology* **187**: 1792–1798.

Salyers, A.A., Whitt, D.D., (null) (2002) *Bacterial pathogenesis*. ASM Press, Washington, D. C. http://www.worldcat.org/title/bacterial-pathogenesis-a-molecular-approach/oclc/47838967.

Seidl, K., Goerke, C., Wolz, C., Mack, D., Berger-Bächi, B., and Bischoff, M. (2008) Staphylococcus aureus CcpA affects biofilm formation. *Infection and Immunity* **76**: 2044–2050.

Senadheera, M.D., Guggenheim, B., Spatafora, G.A., Huang, Y.-C.C., Choi, J., Hung, D.C.I., *et al.* (2005) A VicRK signal transduction system in Streptococcus mutans affects gtfBCD, gbpB, and ftf expression, biofilm formation, and genetic competence development. *Journal of Bacteriology* **187**: 4064–4076.

Serra, D.O., Mika, F., Richter, A.M., and Hengge, R. (2016) The green tea polyphenol EGCG inhibits E. coli biofilm formation by impairing amyloid curli fibre assembly and downregulating the biofilm regulator CsgD via the σ(E) -dependent sRNA RybB. *Molecular Microbiology* **101**: 136–151

Seth, A.K., Geringer, M.R., Nguyen, K.T., Agnew, S.P., Dumanian, Z., Galiano, R.D., *et al.* (2013) Bacteriophage therapy for Staphylococcus aureus biofilm-infected wounds: a new approach to chronic wound care. *Plast Reconstr Surg* **131**: 225–234.

Shanks, R.M.Q., Meehl, M.A., Brothers, K.M., Martinez, R.M., Donegan, N.P., Graber, M.L., *et al.* (2008) Genetic evidence for an alternative citrate-dependent biofilm formation pathway in Staphylococcus aureus that is dependent on fibronectin binding proteins and the GraRS two-component regulatory system. *Infection and Immunity* **76**: 2469–2477.

Shanks, R.M.Q., Sargent, J.L., Martinez, R.M., Graber, M.L., and O'Toole, G.A. (2006) Catheter lock solutions influence staphylococcal biofilm formation on abiotic surfaces. *Nephrol Dial Transplant* **21**: 2247–2255.

Sharma, M., Visai, L., Bragheri, F., Cristiani, I., Gupta, P.K., and Speziale, P. (2008) Toluidine blue-mediated photodynamic effects on staphylococcal biofilms. *Antimicrobial Agents and Chemotherapy* **52**: 299–305.

Shi, S.-F., Jia, J.-F., Guo, X.-K., Zhao, Y.-P., Chen, D.-S., Guo, Y.-Y., and Zhang, X.-L. (2016) Reduced Staphylococcus aureus biofilm formation in the presence of chitosan-coated iron oxide nanoparticles. *Int J Nanomedicine* **11**: 6499–6506.

Sillankorva, S., Oliveira, R., Vieira, M.J., Sutherland, I.W., and Azeredo, J. (2004) Bacteriophage Phi S1 infection of Pseudomonas fluorescens planktonic cells versus biofilms. *Biofouling* **20**: 133–138.

Sinha, B., François, P.P., Nüsse, O., Foti, M., Hartford, O.M., Vaudaux, P., *et al.* (1999) Fibronectin-binding protein acts as Staphylococcus aureus invasin via fibronectin bridging to integrin alpha5beta1. *Cell Microbiol* **1**: 101–117.

Speziale, P., Visai, L., Rindi, S., Pietrocola, G., Provenza, G., and Provenzano, M. (2008) Prevention and treatment of Staphylococcus biofilms. *Curr Med Chem* **15**: 3185–3195.

Stoodley, P., Cargo, R., Rupp, C.J., Wilson, S., and Klapper, I. (2002) Biofilm material properties as related to shear-induced deformation and detachment phenomena. *J Ind Microbiol Biotechnol* **29**: 361–367.

Sugai, M., Komatsuzawa, H., Akiyama, T., Hong, Y.M., Oshida, T., Miyake, Y., *et al.* (1995) Identification of endo-beta-N-acetylglucosaminidase and N-acetylmuramyl-L-alanine amidase as cluster-dispersing enzymes in Staphylococcus aureus. *Journal of Bacteriology* **177**: 1491–1496.

Sutherland, I.W., Hughes, K.A., Skillman, L.C., and Tait, K. (2004) The interaction of phage and biofilms. *FEMS Microbiol Lett* **232**: 1–6.

Switalski, L.M., Patti, J.M., Butcher, W., Gristina, A.G., Speziale, P., and Höök, M. (1993) A collagen receptor on Staphylococcus aureus strains isolated from patients with septic arthritis mediates adhesion to cartilage. *Molecular Microbiology* **7**: 99–107.

Taglialegna, A., Navarro, S., Ventura, S., Garnett, J.A., Matthews, S., Penadés, J.R., *et al.* (2016) Staphylococcal Bap Proteins Build Amyloid Scaffold Biofilm Matrices in Response to Environmental Signals. *PLoS Pathog* **12**: e1005711.

Tamayo, R., Pratt, J.T., and Camilli, A. (2007) Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. *Annu Rev Microbiol* **61**: 131–148.

Tamber, S., and Cheung, A.L. (2009) SarZ promotes the expression of virulence factors and represses biofilm formation by modulating SarA and agr in Staphylococcus aureus. *Infection and Immunity* **77**: 419–428.

Tande, A.J., and Patel, R. (2014) Prosthetic Joint Infection. *Clin Microbiol Rev* **27**: 302–345.

Thomas, V.C., Thurlow, L.R., Boyle, D., and Hancock, L.E. (2008) Regulation of autolysis-dependent extracellular DNA release by Enterococcus faecalis extracellular

proteases influences biofilm development. *Journal of Bacteriology* **190**: 5690–5698.

Tojo, M., Yamashita, N., Goldmann, D.A., and Pier, G.B. (1988) Isolation and characterization of a capsular polysaccharide adhesin from Staphylococcus epidermidis. *J Infect Dis* **157**: 713–722.

Toledo-Arana, A., Merino, N., Vergara-Irigaray, M., Débarbouillé, M., Penadés, J.R., and Lasa, I. (2005) *Staphylococcus aureus* develops an alternative, ica-independent biofilm in the absence of the *arlRS* two-component system. *Journal of Bacteriology* **187**: 5318–5329.

Tormo, M.A., Knecht, E., Götz, F., Lasa, I., and Penadés, J.R. (2005) Bap-dependent biofilm formation by pathogenic species of Staphylococcus: evidence of horizontal gene transfer? *Microbiology (Reading, Engl)* **151**: 2465–2475.

Tormo, M.A., Martí, M., Valle, J., Manna, A.C., Cheung, A.L., Lasa, I., and Penadés, J.R. (2005) SarA is an essential positive regulator of Staphylococcus epidermidis biofilm development. *Journal of Bacteriology* **187**: 2348–2356.

Tormo, M.A., Úbeda, C., Martí, M., Maiques, E., Cucarella, C., Valle, J., *et al.* (2007) Phase-variable expression of the biofilm-associated protein (Bap) in Staphylococcus aureus. *Microbiology (Reading, Engl)* **153**: 1702–1710.

Traber, K.E., Lee, E., Benson, S., Corrigan, R., Cantera, M., Shopsin, B., and Novick, R.P. (2008) agr function in clinical Staphylococcus aureus isolates. *Microbiology (Reading, Engl)* **154**: 2265–2274.

Trotonda, M.P., Manna, A.C., Cheung, A.L., Lasa, I., and Penadés, J.R. (2005) SarA positively controls bap-dependent biofilm formation in Staphylococcus aureus. *Journal of Bacteriology* **187**: 5790–5798.

Trotonda, M.P., Trotonda, M.P., Tamber, S., Memmi, G., and Cheung, A. (2008) MgrA represses biofilm formation in Staphylococcus aureus. *Infection and Immunity* **76**: 1–11.

Truong-Bolduc, Q.C., Dunman, P.M., Strahilevitz, J., Projan, S.J., and Hooper, D.C. (2005) MgrA is a multiple regulator of two new efflux pumps in Staphylococcus aureus. *Journal of Bacteriology* **187**: 2395–2405.

Truong-Bolduc, Q.C., Zhang, X., and Hooper, D.C. (2003) Characterization of NorR protein, a multifunctional regulator of norA expression in Staphylococcus aureus. *Journal of Bacteriology* **185**: 3127–3138.

Tsang, L.H., Cassat, J.E., Shaw, L.N., Beenken, K.E., and Smeltzer, M.S. (2008) Factors contributing to the biofilm-deficient phenotype of Staphylococcus aureus sarA mutants. *PLoS ONE* **3**: e3361.

Tu Quoc, P.H., Genevaux, P., Pajunen, M., Savilahti, H., Georgopoulos, C., Schrenzel, J., and Kelley, W.L. (2007) Isolation and characterization of biofilm formation-defective mutants of Staphylococcus aureus. *Infection and Immunity* **75**: 1079–1088.

Uhlén, M., Guss, B., Nilsson, B., Gotz, F., and Lindberg, M. (1984) Expression of the gene encoding protein A in Staphylococcus aureus and coagulase-negative staphylococci. *Journal of Bacteriology* **159**: 713–719.

Ulrich, M., Bastian, M., Cramton, S.E., Ziegler, K., Pragman, A.A., Bragonzi, A., *et al.* (2007) The staphylococcal respiratory response regulator SrrAB induces *ica* gene transcription and polysaccharide intercellular adhesin expression, protecting *Staphylococcus aureus* from neutrophil killing under anaerobic growth conditions. *Molecular Microbiology* **65**: 1276–1287.

Valle, J., Latasa, C., Gil, C., Toledo-Arana, A., Solano, C., Penadés, J.R., and Lasa, I. (2012) Bap, a biofilm matrix protein of Staphylococcus aureus prevents cellular internalization through binding to GP96 host receptor. *PLoS Pathog* **8**: e1002843.

Valle, J., Toledo-Arana, A., Berasain, C., Ghigo, J.-M., Amorena, B., Penadés, J.R., and Lasa, I. (2003) SarA and not sigmaB is essential for biofilm development by Staphylococcus aureus. *Molecular Microbiology* **48**: 1075–1087.

Valle, J., Vergara-Irigaray, M., Merino, N., Penadés, J.R., Penadés, J.R., and Lasa, I. (2007) sigmaB regulates IS256-mediated Staphylococcus aureus biofilm phenotypic variation. *Journal of Bacteriology* **189**: 2886–96.

Van der Giessen, L.J., Gosselink, R., Hop, W.C.J., and Tiddens, H.A.W.M. (2007) Recombinant human DNase nebulisation in children with cystic fibrosis: before bedtime or after waking up? *Eur Respir J* **30**: 763–768.

Van Schaik, W., and Abee, T. (2005) The role of sigmaB in the stress response of Gram-positive bacteria -- targets for food preservation and safety. *Curr Opin Biotechnol* **16**: 218–224.

Vergara-Irigaray, M., Maira-Litràn, T., Merino, N., Pier, G.B., Penadés, J.R., and Lasa, I. (2008) Wall teichoic acids are dispensable for anchoring the PNAG exopolysaccharide to the Staphylococcus aureus cell surface. *Microbiology (Reading, Engl)* **154**: 865–877.

Vergara-Irigaray, M., Valle, J., Merino, N., Latasa, C., García, B., Ruiz de Los Mozos, I., *et al.* (2009) Relevant Role of Fibronectin-Binding Proteins in Staphylococcus aureus Biofilm-Associated Foreign-Body Infections. *Infection and Immunity* **77**: 3978–3991.

Villanueva, M., García, B., Valle, J., Rapún, B., Ruiz de Los Mozos, I., Solano, C., *et al.* (2018) Sensory deprivation in Staphylococcus aureus. *Nat Commun* **9**: 523.

Vuong, C., Kocianova, S., Voyich, J.M., Yao, Y., Fischer, E.R., DeLeo, F.R., and Otto, M. (2004) A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. *J Biol Chem* **279**: 54881–54886.

Vuong, C., Saenz, H.L., Gotz, F., and Otto, M. (2000) Impact of the agr quorum-sensing system on adherence to polystyrene in Staphylococcus aureus. *J Infect Dis* **182**: 1688–1693.

Vuong, C., Voyich, J.M., Fischer, E.R., Braughton, K.R., Whitney, A.R., DeLeo, F.R., and Otto, M. (2004) Polysaccharide intercellular adhesin (PIA) protects Staphylococcus epidermidis against major components of the human innate immune system. *Cell Microbiol* **6**: 269–275.

Wertheim, H.F., Melles, D.C., Vos, M.C., van Leeuwen, W., van Belkum, A., Verbrugh, H.A., and Nouwen, J.L. (2005) The role of nasal carriage in Staphylococcus aureus infections. *Lancet Infect Dis* **5**: 751–762.

Whitchurch, C.B., Tolker-Nielsen, T., Ragas, P.C., and Mattick, J.S. (2002) Extracellular DNA required for bacterial biofilm formation. *Science* **295**: 1487–1487.

Whitehouse, J.D., Friedman, N.D., Kirkland, K.B., Richardson, W.J., and Sexton, D.J. (2002) The impact of surgical-site infections following orthopedic surgery at a community hospital and a university hospital: adverse quality of life, excess length of stay, and extra cost. *Infect Control Hosp Epidemiol* **23**: 183–189.

Winkler, M.E., and Hoch, J.A. (2008) Essentiality, Bypass, and Targeting of the YycFG (VicRK) Two-Component Regulatory System in Gram-Positive Bacteria. *Journal of Bacteriology* **190**: 2645–2648.

Wittebole, X., De Roock, S., and Opal, S.M. (2014) A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens. *Virulence* **5**: 226–235.

Wu, J.A., Kusuma, C., Mond, J.J., and Kokai-Kun, J.F. (2003) Lysostaphin disrupts Staphylococcus aureus and Staphylococcus epidermidis biofilms on artificial surfaces. *Antimicrobial Agents and Chemotherapy* **47**: 3407–3414.

Wu, Y., Wang, J., Xu, T., Liu, J., Yu, W., Lou, Q., *et al.* (2012) The Two-Component Signal Transduction System ArlRS Regulates Staphylococcus epidermidis Biofilm Formation in an ica-Dependent Manner. *PLoS ONE* **7**: e40041.

Yang, S.-J., Dunman, P.M., Projan, S.J., and Bayles, K.W. (2006) Characterization of the Staphylococcus aureus CidR regulon: elucidation of a novel role for acetoin metabolism in cell death and lysis. *Molecular Microbiology* **60**: 458–468.

Yarwood, J.M., Bartels, D.J., Volper, E.M., and Greenberg, E.P. (2004) Quorum Sensing in Staphylococcus aureus Biofilms. *Journal of Bacteriology* **186**: 1838–1850.

Yilmaz, C., Colak, M., Yilmaz, B.C., Ersoz, G., Kutateladze, M., and Gozlugol, M. (2013) Bacteriophage therapy in implant-related infections: an experimental study. *J Bone Joint Surg Am* **95**: 117–125.

Yousif, A., Jamal, M.A., and Raad, I. (2015) *Biofilm-Based Healthcare-associated Infections: Volume I*. Springer International Publishing, Cham.

Zhang, L., and Ji, G. (2004) Identification of a staphylococcal AgrB segment(s) responsible for group-specific processing of AgrD by gene swapping. *Journal of Bacteriology* **186**: 6706–6713.

Zhao, L., Hu, Y., Xu, D., and Cai, K. (2014) Surface functionalization of titanium substrates with chitosan-lauric acid conjugate to enhance osteoblasts functions and inhibit bacteria adhesion. *Colloids Surf B Biointerfaces* **119**: 115–125.

Ziebuhr, W., Krimmer, V., Rachid, S., Lössner, I., Gotz, F., and Hacker, J. (1999) A novel mechanism of phase variation of virulence in Staphylococcus epidermidis: evidence for control of the polysaccharide intercellular adhesin synthesis by alternating insertion and excision of the insertion sequence element IS256. *Molecular Microbiology* **32**: 345–356.

OBJETIVOS

OBJETIVOS

Como ya ha quedado de manifiesto a lo largo de la introducción de esta tesis, las infecciones causadas por la formación de biofilms sobre implantes médicos representa un inmenso problema clínico. Aunque actualmente se están estudiando y utilizando diversas estrategias para hacerles frente, a día de hoy no se ha encontrado una solución satisfactoria para evitar o tratar infecciones causadas por bacterias en biofilm.

En esta tesis, hemos abordado este problema y nos hemos fijado en las distintas etapas de la formación del biofilm de *Staphylococcus aureus*, para desarrollar estrategias que eviten la adhesión bacteriana o la formación de la matriz del biofilm.

Por ello los objetivos específicos de esta tesis son**:**

1.- Evaluar si la modificación de la topografía de la superficie es una estrategia eficaz para prevenir la formación de biofilms por *S. aureus* sobre implantes médicos.

2.- Identificar que sistemas de dos-componentes de *S. aureus* son importantes para la colonización de catéteres implantados subcutáneamente en un modelo de infección murino, con el fin de utilizarlos como posibles dianas para bloquear el proceso de formación del biofilm.

3.- Evaluar el potencial de las proteínas de la matriz del biofilm como componente vacunal para inducir una respuesta inmune protectora frente infecciones por biofilm de *S. aureus*.

CHAPTER I

Evaluation of Surface Microtopography Engineered by Direct Laser Interference for Bacterial Anti-Biofouling

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CHAPTER II

A systematic evaluation of the two-component systems network reveals that ArlRS is a key regulator of catheter colonization by Staphylococcus aureus

ABSTRACT

Two-component systems (TCS) are modular signal transduction pathways that allow cells to adapt to prevailing environmental conditions by modifying cellular physiology. *Staphylococcus aureus* has sixteen TCSs to adapt to the diverse microenvironments encountered during its life cycle, including host tissues and implanted medical devices. *S. aureus* is particularly prone to cause infections associated to medical devices, whose surfaces coated by serum proteins constitute a particular environment. Identification of the TCSs involved in the adaptation of *S. aureus* to colonize and survive on the surface of implanted devices remains largely unexplored. Here, using an *in vivo* catheter infection model and a collection of mutants in each nonessential TCS of *S. aureus,* we investigated the requirement of each TCS for colonizing the implanted catheter. Among the fifteen mutants in nonessential TCSs, the *arl* mutant exhibited the strongest deficiency in the capacity to colonize implanted catheters. Moreover, the *arl* mutant was the only one presenting a major deficit in PNAG production, the main exopolysaccharide of the *S. aureus* biofilm matrix whose synthesis is mediated by the *icaADBC* locus. Regulation of PNAG synthesis by ArlRS occurred through repression of IcaR, a transcriptional repressor of *icaADBC* operon expression. Deficiency in catheter colonization was restored when the *arl* mutant was complemented with the *icaADBC* operon. MgrA, a global transcriptional regulator downstream ArlRS that accounts for a large part of the *arlRS* regulon, was unable to restore PNAG expression and catheter colonization deficiency of the *arlRS* mutant. These findings indicate that ArlRS is the key TCS to biofilm formation on the surface of implanted catheters and that activation of PNAG exopolysaccharide production is, among the many traits controlled by the ArlRS system, a major contributor to catheter colonization.

INTRODUCTION

The extensive use of indwelling devices such as prosthetic joints, heart valves and intravascular catheters in hospitalized patients has increased the incidence of device related infections (DRI) (Hogan *et al.*, 2015). DRI constitute a growing healthcare concern because they are associated with a high morbidity and mortality and also with increased costs in health care systems. *Staphylococcus aureus* is a frequent etiological agent of DRI, especially those related with intravascular catheters, prosthetic joints, vascular grafts and pacemakers (Kluytmans *et al.*, 1997; Lowy, 1998; Ellis *et al.*, 2014).

The success of *S. aureus* as a pathogen depends on the combined action of multiple factors. On one hand, *S. aureus* produces a wide array of cell surface and secreted virulence factors such as exoenzymes (coagulase, lipases and proteases), toxins (cytolytic toxins, enterotoxins, exfoliative toxins) and immune evasion mechanisms (protein A) (Foster *et al.*, 2014). Cell surface virulence factors allow the organism to colonize the host through adhesion to mucosal surfaces and to interfere with normal immune system functions. Secreted factors, including exoenzymes and exotoxins, allow the organism to spread into surrounding tissues and access nutrients through cell damage. On the other hand, *S. aureus* exhibits a great capacity to adapt to many different environments including almost every organ of the human body (lung, heart, blood, bone, skin, muscles, eye, joints and intestinal tract). For that, *S. aureus* possesses efficient signal transduction systems that facilitate the integration of environmental stimuli and adjust the cellular physiology in response (Novick, 2003; Cheung *et al.*, 2004; Haag and Bagnoli, 2016). The sensory machinery of *S. aureus* is composed of the two-component systems (TCS) network, a Ser/Thr protein kinase, two Ser/Thr phosphatases and a c-di-AMP cyclase (Dac) and a phosphodiesterase (GpdP) involved in c-di-AMP metabolism. As regards the TCS network, most *S. aureus* strains contain 16 TCSs, with one of them being essential for bacterial viability (Kuroda *et al.*, 2001). In a canonical TCS, extracellular stimuli induce the autophosphorylation of a histidine kinase (HK). The activated HK transfers the phosphoryl group to a conserved aspartate residue present on the response regulator (RR). The phosphorylation of the RR activates an output domain, which can then effect changes in cellular physiology often by regulating gene expression (Novick, 2003; Haag and Bagnoli, 2016).

When *S. aureus* reaches the surface of an implanted medical device, attachment to the surface occurs through interactions between bacterial adhesins and plasma proteins deposited on the implant's surface. Once attached, *S. aureus* needs to adjust

bacterial physiology to the sessile lifestyle. For that, *S. aureus* produces an extracellular matrix mainly composed of exopolysaccharides, proteins and extracellular DNA (O'Gara, 2007; Rice *et al.*, 2007). The exopolysaccharide PNAG, also named PIA (polysaccharide intercellular adhesin) is a major component of the staphylococcal biofilm matrix. PNAG, β-1,6-linked N-acetylglucosamine, is synthetized by the enzymes encoded in the *icaADBC* operon whose expression is tightly regulated by the transcriptional repressor IcaR. Some strains of *S. aureus* make use of extracellular proteins to build the biofilm matrix by interacting with eDNA and polysaccharides or in some cases, through polysaccharide-independent mechanisms (Cucarella *et al.*, 2001; Corrigan *et al.*, 2007; O'Neill *et al.*, 2008; Vergara-Irigaray *et al.*, 2009; Taglialegna *et al.*, 2016). Although the molecular determinants underlying the choice of either a polysaccharide or protein-based biofilm matrix are not well understood, it is assumed that environmental signals determine the composition of the biofilm matrix (Vergara-Irigaray *et al.*, 2009). Attempts to identify the specific TCSs involved in the biofilm formation process of *S. aureus in vitro* have been previously performed (Toledo-Arana, *et al.*, 2005). However, identification of TCSs important for colonization of implanted medical devices *in vivo* remains to be elucidated.

In this study, we used a collection of single mutants in each nonessential TCS of *S. aureus* and a murine subcutaneous catheter infection model to identify the TCSs controlling the production and composition of the biofilm matrix on the surface of the implanted device. Our results revealed that *arlRS*, and in a lower extent *agr* and *srrAB* mutants, have a significant decreased capacity in colonize the surface of implanted catheters. In the *arl* mutant background, expression of the *icaADBC* operon restores the capacity to colonize the catheter, indicating that the PNAG exopolysaccharide is required for efficient colonization of implanted catheters. This result is central to the development of novel therapeutic approaches to specifically target biofilm related infections.

MATERIALS AND METHODS

Oligonucleotides, plasmids, bacterial strains and culture conditions

Bacterial strains, plasmids and oligonucleotides (Stabvida) used in this study are listed in **Table S1** and **Table S2**. *Escherichia coli* strains were grown in LB broth (Conda-Pronadisa). *S. aureus* strains were grown in trypticase soy broth (TSB) (Conda-Pronadisa), TSB supplemented with glucose (TSBg) in strain ISP479r or NaCl 3% (TSB-NaCl) in the case of strain 132. When required for selection, medium was supplemented with appropriate antibiotics at the following concentrations: erythromycin (Em), 1.5 μg ml^{-1} and 10 μg ml^{-1}; ampicillin (Amp), 100 μg ml^{-1}.

Table S1. Strains and plasmids used in this study

Strains	Description	MIC	Reference
Staphylococcus			
MW2	Community-acquired strain of MRSA, which was isolated in 1998 in North Dakota, USA.	3566	(Baba *et al.*, 2008)
132	MRSA clinical strain	29	(Vergara-Irigaray *et al.*, 2009)
Isp479r	ISP479c with rsbU gene restored	1680	(Toledo-Arana, *et al.*, 2005)
MW2 hpt	MW2 ΔhptRS	4032	(Villanueva *et al.*, 2018)
MW2 lyt	MW2 ΔlytSR	2964	(Villanueva *et al.*, 2018)
MW2 gra	MW2 ΔgraRS	11	(Villanueva *et al.*, 2018)
MW2 sae	MW2 ΔsaeRS	2965	(Villanueva *et al.*, 2018)
MW2 tcs7	MW2 ΔMW1208-MW1209	4033	(Villanueva *et al.*, 2018)
MW2 arl	MW2 ΔarlRS	4034	(Villanueva *et al.*, 2018)
MW2 srr	MW2 ΔsrrAB	2966	(Villanueva *et al.*, 2018)
MW2 pho	MW2 ΔphoPR	4035	(Villanueva *et al.*, 2018)
MW2 air	MW2 ΔairSR	3670	(Villanueva *et al.*, 2018)
MW2 vra	MW2 ΔvraSR	4036	(Villanueva *et al.*, 2018)
MW2 agr	MW2 ΔagrBDCA	4037	(Villanueva *et al.*, 2018)
MW2 kdp	MW2 ΔkdpDE	4038	(Villanueva *et al.*, 2018)
MW2 hss	MW2 ΔhssRS	2979	(Villanueva *et al.*, 2018)
MW2 nre	MW2 ΔnreBC	2967	(Villanueva *et al.*, 2018)
MW2 bra	MW2 ΔbraRS	4039	(Villanueva *et al.*, 2018)
MW2 arl+parlRS	MW2 Δarl carrying pCN51::arlRS plasmid	4539	(Villanueva *et al.*, 2018)
MW2 arl Δica+parlRS	MW2 Δarl with a deletion of the ica operon carryingpCn51::arlRS plasmid	6642	This study
132 arl	132 with a deletion of the arlRS genes	6050	This study
132 arl parlRS	132 Δarl carrying pCN51::arlRS plasmid	6568	This study
Isp479r arl	ISP479r with a deletion of the arlRS genes	1921	(Toledo-Arana, *et al.*, 2005)
Isp479r arl parlRS	Isp479r Δarl carrying pCN51::arlRS plasmid	6569	This study
132 Pica(BS)-gfp	132 carrying pCN52::Pica(BS)-gfp plasmid	6011	This study
132 arl Pica(BS)-gfp	132 Δarl carrying pCN52::Pica(BS)-gfp plasmid	6639	This study
Isp479r Pica(BS)-gfp	Isp479r carrying pCN52::Pica(BS)-gfp plasmid	6032	This study
Isp479r arl Pica(BS)-gfp	Isp479r Δarl carrying pCN52::Pica(BS)-gfpplasmid	6640	This study
132 IcaC_3xflag	32 carrying a 3xFLAG epitope at icaC	2731	This study
132 arl IcaC_3xflag	132 Δarl carrying a 3xFLAG epitope at icaC	6547	This study
132 arl IcaC_3xflag+parlRS	132 Δarl carrying a 3xFLAG epitope at icaC and pCN51::arlRS plasmid	6548	This study
Isp479r IcaC_3xflag	Isp479r carrying a 3xFLAG epitope at icaC	2641	This study
Isp479r arl IcaC_3xflag	Isp479r Δarl carrying a 3xFLAG epitope at icaC	6549	This study
Isp479r arl IcaC_3xflag+parlRS	Isp479r Δarl carrying a 3xFLAG epitope at icaC and pCN51::arlRS plasmid	6550	This study
MW2 Δica	MW2 with a deletion of the ica operon	6367	This study
MW2 PCd-ica	MW2 expressing the ica operon under de cadmium inducible promoter	6370	This study
MW2 arl PCd-ica	MW2 Δarl expressing the ica operon under de cadmium inducible promoter	6546	This study
MW2 Pmgra-gfp	MW2 carrying pCN52::PmgrA-gfp plasmid	5990	This study
MW2 arl Pmgra-gfp	MW2 Δarl carrying pCN52::PmgrA-gfp plasmid	5991	This study

Strains	Description	MIC	Reference
MW2 mgrA	MW2 with a deletion of the mgrA gene	6643	This study
MW2 arl PCd-mgrA	MW2 Δarl expressing the mgrA gene under de cadmium inducible promoter	6644	This study
MW2 mgrA PCd-ica	MW2 ΔmgrA expressing the ica operon under de cadmium inducible promoter	6645	This study
MW2 ebh	MW2 with a deletion of the first 970 bp of the ebh mRNA	6646	This study
MW2 spa	MW2 with a deletion of the spa gene	5003	This study
MW2 sasG	MW2 with a deletion of the sasG gene	6647	This study

Plasmids	Description	Reference
pMAD	E. coli-S. aureus shuttle vector with a thermosensitive origin of replication for gram-positive bacteria	(Arnaud *et al.*, 2004)
pMAD::TCS3AD	pMAD plasmid containing the allele for deletion of the hptRS genes	(Villanueva *et al.*, 2018)
pMAD::TCS4AD	pMAD plasmid containing the allele for deletion of the lytSR genes	(Villanueva *et al.*, 2018)
pMAD::TCS5AD	pMAD plasmid containing the allele for deletion of the graRS genes	(Villanueva *et al.*, 2018)
pMAD::TCS6AD	pMAD plasmid containing the allele for deletion of the saeRS genes	(Toledo-Arana, *et al.*, 2005)
pMAD::TCS7AD	pMAD plasmid containing the allele for deletion of the MW1208-MW1209 genes	(Villanueva *et al.*, 2018)
pMAD::TCS8AD	pMAD plasmid containing the allele for deletion of the arlRS genes	(Toledo-Arana, et al., 2005)
pMAD::TCS9AD	pMAD plasmid containing the allele for deletion of the srrAB genes	(Villanueva et al., 2018)
pMAD::TCS10AD	pMAD plasmid containing the allele for deletion of the phoPR genes	(Toledo-Arana, et al., 2005)
pMAD::TCS11AD	pMAD plasmid containing the allele for deletion of the airSR genes	(Villanueva et al., 2018)
pMAD::TCS12AD	pMAD plasmid containing the allele for deletion of the vraSR genes	(Toledo-Arana, et al, 2005)
pMAD::TCS13AD	pMAD plasmid containing the allele for deletion of the agrBDCA genes	(Valle et al., 2003)
pMAD::TCS14AD	pMAD plasmid containing the allele for deletion of the kdpED genes	(Villanueva et al., 2018)
pMAD::TCS15AD	pMAD plasmid containing the allele for deletion of the hssRS genes	(Toledo-Arana, et al., 2005)
pMAD::TCS16AD	pMAD plasmid containing the allele for deletion of the nreBC genes	(Villanueva et al., 2018)
pMAD::TCS17AD	pMAD plasmid containing the allele for deletion of the braRS genes	(Villanueva et al., 2018)
pCN51	E. coli - S. aureus shuttle vector to express genes under the control of the Pcad cadmium-inducible promoter. EmR	(Charpentier et al., 2004)
pCN51::arlRS	pCN51 plasmid expressing arlRS genes	(Villanueva et al., 2018)
pCN52	E. coli - S. aureus shuttle vector with promoterless gfpmut2 fusion vector. EmR	(Charpentier et al., 2004)
pCN52::Pica(BS)-gfp	pCN52 plasmid expressing gfpmut2 under the ica promoter	This study
pCN52::PmgrA-gfp	pCN52 plasmid expressing gfpmut2 under the mgrA promoter	This study
pMAD::icaAD	pMAD plasmid containing the allele for deletion of 2684 bp of the ica operon	(Toledo-Arana, et al., 2005)
pMAD::icaC_3xflag	pMAD_lic plasmid containing the allele for insertion of the 3xflag epitope after the icaC gene	This study
pMAD_lic	pMAD modified plasmid for insert DNA fragments using a ligase independent cloning	This study
pMAD_lic::PCd_ica	pMAD_lic plasmid containing the allele for insertion of the cadmium-inducible promoter before the icaADBC mRNA	This study
pMAD_lic::mgrA-AD	pMAD_lic plasmid containing the allele for deletion of the mgrA gene	This study
pMAD_lic::PCd_mgrA	pMAD_lic plasmid containing the allele for insertion of the cadmium-inducible promoter before the mgrA mRNA	This study
pMAD_lic::ebh-AD	pMAD_lic plasmid containing the allele for deletion of the first 970 bp of ebh mRNA	This study
pMAD::spa-AD	pMAD plasmid containing the allele for deletion of the spa gene	(Merino et al., 2009)
pMAD::sasG-AD	pMAD plasmid containing the allele for deletion of the sasG gene	(Vergara-Irigaray et al., 2009)

Table S2. Oligonucleotides used in this study

Oligonucleotide	*Sequence 5´→3´*
Deletion of two-component systems	
hpt-A (BamHI)	GGATCCGTTATCAGAAACAATTGATCA
hpt-B (NcoI)	CCATGGTGAATCATCTCCAAAAATTTAT
hpt-C (NcoI)	CCATGGATTAGCACATAACTAATGATTA
hpt-D (XhoI)	CTCGAGTAAAGGCGATGCAGTTAATA
hpt-E	ATCTACTCAAGTACTGCTTT
hpt-F	TGTTTCAAACGTCTTTGTATA
lyt-A (BamHI)	GGATCCTGACGTTGAACAAACGAATA
lyt-B (HindIII)	AAGCTTGATAGCACCTCAGTAAAT
lyt-C (HindIII)	AAGCTTCAGTAATCCTTTTTTTTATGC
lyt-D (EcoRI)	GAATTCCAACTGTACCGATACCAAT
lyt-E	GAAAAGAAAAATGTAGATTTGA
lyt-F	AATAACATCATTGGCAAATTG
gra-A (BamHI)	GGATCCCAAATAGATATTGCTGTATTC
gra-B (NcoI)	CCATGGCCATATCACCCAATATCATT
gra-C (NcoI)	CCATGGACATGCGTTTTGTTACTTAG
gra-D (XhoI)	CTCGAGTTAACGCCACCTAAAACAC
gra-E	AAATGACTTGGATTCAGTGT
gra-F	AAAAAGATAGATGGCATAATG
sae-A	ATTTGAATGGATAGGC
sae-B	ATTACGTCATAATCCG
sae-C	AAATCGGATTATGACGTAATAAGTGGGTCATCTATTTTTTCACC
sae-D	TAACACTACAAATCGC
sae-E	GTTGGTGATTTTAGACTTTTA
sae-F	ATAAGATTGTAGAGCACATAA
tcs7-A (BamHI)	GGATCCAAAGGGGATCTAATTATGATA
tcs7-B (HindIII)	AAGCTTATTTTATTCCGCCCTTTTAAA
tcs7-C (HindIII)	AAGCTTATACAAACAAAAAAGTATTGAG
tcs7-D (EcoRI)	GAATTCAAAAATACTTCTCTCTAGCAA
tcs7-E	AATTGCCGGAAGATGTTAAA
tcs7-F	TATCTTTGTAGGCTTTATCG
arl-A	GTTTCCTTTTTGGAGG
arl-B	TCATGACTGAGACGTC
arl-C	GATTGACGTCTCAGTCATGAGCGTCATTTGTACACC
arl-D	GTGATGAGGTTTAAAC
arl-E	AGTGATCTGAAACAATTCC
arl-F	AAGAATAGTAAATAAAACGC
srr-A (BamHI)	GGATCCCCTACAACATTTGTAGCTTT
srr-B (XhoI)	CTCGAGAAACTACCAAAACCAGAATAA
srr-D (BamHI)	GGATCCCATTTTATTCCATAAACCAAC
srr-E	TTTGTACAAAGGTAGACTTG
srr-F	GGTATTGAATTAGAAGATGG
pho-A	TGGTAGTAAGATACCC
pho-B	AAAGTGGTAACAGCGC

Oligonucleotide	Sequence 5´→3´
pho-C	ACACGCGCTGTTACCACTTTGGTATGCCTCCCTAAC
pho-D	CACATGGTACAGCTCC
pho-E	AGATATTTCCGATATAGCGA
pho-F	CAGGTGCAAACATTAATTATG
air-A	TTAAAATGTGTGTAATTGCA
air-B (HindIII)	AAGCTTCAAATCGCTCCAATTCATTT
air-C (HindIII)	AAGCTTAATGAGCTTTTAAATATTTGTC
air-D	GTGTGTTACATCGCTTTTA
air-E	CATATAAAGGATCACCAATAA
air-F	CATAGTTATTCATTATACCAC
vra-A	GTATTACCAGGTGCAG
vra-B	TCAATAGTTCGTATTG
vra-C	AATTCAATACGAACTATTGACGATAAATCACCTCTA
vra-D	ACGTGGCCTTTTGGCG
vra-E	TGACGAACAAGTGAAATGG
vra-F	CGTTCTATTATTGGGATGTG
agr-A	AGCACTGAGTCCAAGG
agr-B	TTTTACACCACTCTCC
agr-C	GTGAGGAGAGTGGTGTAAAAAAGATAATAAAGTCAGTTAACGGC
agr-D	CAGTTATTAGCAGGAT
agr-E	GGGGATGTTATTAATTATGAA
agr-F	TAGTCATTTATACGAAGGGA
kdp-A (BamHI)	GGATCCCCAATGATATTAGTTAATCCA
kdp-B (NcoI)	CCATGGAACCTTCACCTCGATAGC
kdp-C (NcoI)	CCATGGCACAATGTCATGAGGACG
kdp-D (EcoRI)	GAATTCGTTTTCAATAATTGATTCTCTG
kdp-E	TACTAATTAAACATGATAATGG
kdp-F	GAATTCGTTTTCAATAATTGATTCTCTG
hss-A	CATTAATAGCGACCTC
hss-B	CTCCCTTATCTTTTTC
hss-C	AAATGAAAAAGATAAGGGAGTCTCTACCTCCTGAAA
hss-D	AGGTGTAGTGTGCATC
hss-E	CATACATTGTGTCGTTTAAAA
hss-F	AACCAATGATTAAGCTAATAAA
nre-A (BamHI)	GGATCCCGCAACATTAGTAACCAATAT
nre-B (NcoI)	CCATGGGACTTACACCCTAATTCATC
nre-C (NcoI)	CCATGGAGTTTGAAATTAATATAATTCAGT
nre-D (XhoI)	CTCGAGTTATAAACAGCAAGACTTAGAA
nre-E	TTAAGTTCAGCGTCGGATAT
nre-F	AACTTTACATTATTACGATGAAA
bra-A (BamHI)	GGATCCGCTGCAGAATCAGTAATATT
bra-B (HindIII)	AAGCTTCTATAATCTTCTTCCTTCAAT
bra-C (HindIII)	AAGCTTAAACTTTCAATATTGTAAGCATA
bra-D (EcoRI)	GAATTCGTGCCATAACAATCTTAACT
bra-E	TACTTTCTGCTTGTTACTGT
bra-F	ACACAAGCGTATATTCAATC

Oligonucleotide	Sequence 5´→3´
Constitutive expression of *arlRS*	
ArlR-Fw (SalI XhoI)	GTCGACATTGCTCGAGGTAATATGAGGTGTACAAAT
ArlS-Rv (XmaI AscI)	GGCGCGCCCGGGGGATTAAAATATGATTTTAAACG
pMAD_lic	
pMAD_lic (EcoRI)	GAATTCTGACGACGACAAGAGGGCCC
pMAD_lic (BamHI)	GGATCCTGAGGAGAAGCCCGGGGCCC
Transcriptional expression of *ica* and *mgra* promoters	
AU59	ATGCCTGCAGGTCGACTTTTTATAACCCCCTACTGAAAATTAATCACACT
AU76	ACGAATTCGAGCTCGGTACCTTTCTTTACCTACCTTTCGTTAGTTAGGTTG
PmgrA-fw	ATGCCTGCAGGTCGACGTCCCCTTTTAAAGCAATGGC
PmgrA-rv	ACGAATTCGAGCTCGAATAAGAATATCCATAATTAACGGATTTTTGGGTAGT
Deletion of *ica*	
AU6 Ica-A	CACACTATGTTACAGGAA
AU7 Ica-B	GGATCCGCACCAAGTTTTGGATCA
AU8 Ica-C	GGATCCGGGTATTTGCACGCATTT
Ica-D	TCAAGTAATATCTTGGCG
IcaC 3xflag epitope insertion	
CFLAG-A	GTCGACGCAAATGGAGACTATTG
CFLAG-B	GCGGCCGCTTACTATTTATCGTCGTCATCTTTGTAGTCGATATCATGATCTTT ATAATCACCGTCATGGTCTTTGTAGTCATAAGCATTAATGTTCAATTTA
CFLAG-C	GCGGCCGCTTATTAAGCTATGTTAAAAAC
CFLAG-D	CCATGGGCACAAGAGAAGAATTAC
CFLAG-E	TCACGATACCGTGCTACA
CFLAG-F	CACACGCATCAGATTTAG
Cadmium promoter insertion before *icaADBC* operon	
Pica_LIC-A	GACGACGACAAGAGTCTTATTCTTTTCAGGGGAAC
PCd-ica-B	TGAATAAGTGCGTTTCTTTACCTACCTTTCGT
Ica-Cd-fw	TAGGTAAAGAAACGCACTTATTCAAGTGTATTTTT
Ica-Cd-rv	AAAAAATTGCAATGCAGGTTCAGACATTG
PCd-ica-C	TCTGAACCTGCATTGCAATTTTTTAACTTTTTGC
Pica_LIC-D	GAGGAGAAGCCCGGTCTTGATCAACGATAGTATCTG
Cadmium promoter insertion before *mgrA* gene	
PmgrA_LIC-A	GACGACGACAAGAGTCGTCCCCTTTTAAAGCAATG
PCd-mgrA-B	AATAAGTGCGTAAAGTTCTCCTCCAGACATAC
mgrA-Cd-fw	GGAGAACTTTACGCACTTATTCAAGTGTATTTTT
mgrA-Cd-rv	AATAAGTGCGTAAAGTTCTCCTCCAGACATAC
PCd-mgrA-C	GAACCTGCAATGTCTGATCAACATAATTTAAAAG
PmgrA_LIC-D	GAGGAGAAGCCCGGTTTATTTTTCCTTTGTTTCATCAAATG
Deletion of mgra, ebh, sasG and spa genes	
mgrA LIC-A	GGAGGAGAACTTTATTAACTTTTGTCATGACAATTAAAGTAATG
mgra-B	ATGACAAAAGTTAATAAAGTTCTCCTCCAGACATACTAT
mgra-C	GGAGGAGAACTTTATTAACTTTTGTCATGACAATTAAAGTAATG
mgrA LIC-D	GAGGAGAAGCCCGGTGGCACTAGAACGTCAAATTGAC
mgrA-E	GACATGCAACTAGTAATTCCA
mgrA-F	GATGAAAAAGATGAAGCGGT
Ebh-LIC-A	GACGACGACAAGAGTTAGGTTATTTATTCTTTGGTTTAGGAC

Oligonucleotide	Sequence 5´→3´
Ebh-B	ATTCTGTTTGAGCTCCAGAATATAATAACACAAAATATATT
Ebh-C	TTATATTCTGGAGCTCAAACAGAATTCAAACGCG
ebh-LIC-D	GAGGAGAAGCCCGGTTCCATATTCCCACATTTGTTGA
ebh-E	GAGTTCTTATTGTCGGAGAATG
ebh-F	TGGCCACTTCTATTTCTTATTTT
sasG-A	GGATCCATTGAATGCATCCACCAGC
sasG-B	AAGCTTCTAAAGAATCAGTAGCTAATC
sasG-C	AAGCTTTGATGGTTCATGTTCGATTG
sasG-D	GAGATTTTAATATCTTGGTGC
sasG-E	TGTAACGAAAACAGCACAAAG
sasG-F	TGTAGAGACCCACATGATAGTAGA
spa-A	GAATTCCAATTCTAGCTATTATCACTT
spa-B	TCTAGAATTAATACCCCCTGTATGTA
spa-C	TCTAGAAAACAAACAATACACAACGAT
spa-D	GGATCCTTAAAATGGAAAAGTGCAGG
spa-E	GATGATGTATACAATGTATTTC
spa-F	TGCGTCTCGATTTAATTGG
Riboprobes	
AU52	TAATACGACTCACTATAGGGTATCCACGTAAATGCAATTTCC
AU53	TGGAAGTTCAGATAATACAGC
AU54	TAATACGACTCACTATAGGGGAATTCACGCAATATCAT
AU55	TCACGATACCGTGCTACAC
RP T7 sense icaR	TAATACGACTCACTATAGGGTACTTTCTTCCACTGCTCCA
icaR +1 (BamHI)	GGATCCGAAATATTTGTAATTGCAACTTA

DNA manipulations and bacterial transformation

General DNA manipulations were performed using standard procedures. Plasmids were purified using the NucleoSpin Plasmid miniprep kit (Macherey-Nagel) according to the manufacturer´s protocol. FastDigest restriction enzymes and Rapid DNA ligation kit (Thermo Scientific) were used according to the manufacturer´s instructions. Plasmids were transformed into *E. coli XL1-Blue* strain (Stratagene) and *S. aureus* by electroporation, using previously described protocols (Cucarella *et al.*, 2001). Staphyloccocal electrocompetent cells were generated as previously described (Schenk and Laddaga, 1992).

Allelic exchange of chromosomal genes

We used a collection of single mutants in each TCS constructed in *S. aureus* MW2 (listed in **Table S1**) by as follows. Fragments of at least 500 bp that flanked the left (primers A and B) and right sequences (primers C and D) of the region targeted for deletion were amplified by PCR. The PCR products AB and CD were used to obtain an overlapping PCR product named AD, which was cloned into the shuttle vector pMAD (Arnaud *et al.*, 2004)or pMAD_lic. A LIC-modified pMAD vector was constructed in order to enable efficient directional cloning without restriction enzyme digestion or ligation reactions (Aslanidis and de Jong, 1990). To create the pMAD_lic vector we oligomerized the primers pMAD_lic (EcoRI) and pMAD_lic (BamHI) listed in **Table S2**, and cloned the double DNA strand dimer into pMAD using EcoRI and BamHI restriction enzymes. To produce specific non-complementary overhangs in the pMAD_lic vector, the ApaI linearized plasmid was treated with T4 DNA Polymerase (Novagen) in the presence of dTTP (Novagen) for 30 min at 22ºC. PCR products with complementary overhangs were created using Phusion enzyme (Thermo Scientific) by building appropriate 5' extensions into the primers. The PCR products were purified and then treated with T4 DNA Polymerase in the presence of dATP (Novagen) at 22ºC. After 30 min the enzyme was inactivated. To anneal the insert into the pMAD_lic vector, the mix of vector and insert was incubated for 5 min at 22ºC and then, EDTA (6.25 mM) was added and a further incubation of 5 min at 22ºC was applied. To express the *ica* operon and *mgrA* gene under de cadmium promoter, we amplified the cadmium inducible promoter from pCN51 plasmid (P_{cd}) and two fragments of at least 500 bp that flanked the left (primers A and B) and right sequences (primers C and D) of the start of the mRNA of both genes. Three fragments were fused by overlapped PCR. The purified

fragment was cloned into the pMAD_lic plasmid. Allelic exchange in the absence of a selection marker was performed as previously described (Valle *et al.*, 2003). White colonies, which no longer contained the pMAD plasmid, were tested to confirm the replacement by PCR using primers E and F (**Table S2**). Note that cadmium was not used for the expression of P_{cd} since the leakage of expression of the P_{cd} promoter was sufficient to express PNAG and MgrA.

PNAG detection

Overnight cultures of the strains tested were diluted 1:40 in the appropriate medium, and 2 ml of this cell suspension was used to inoculate sterile 24-well polystyrene microtiter plates (Sarstedt). After 16 h cell-surface PNAG production was detected as described previously (Valle *et al.*, 2003). 5 µl of the extract or 5 µl of a dilution of the purified extract were spotted on a nitrocellulose filter using a Bio-Dot microfiltration apparatus (Bio-Rad), blocked overnight with 5% skim milk in phosphate-buffered saline with 0.1% Tween 20, and incubated for 2 hours with an anti-PNAG antibody diluted 1:10,000 (Maira-Litràn *et al.*, 2002). Bound antibodies were detected with peroxidase-conjugated goat anti-rabbit immunoglobulin G anti-bodies (Invitrogen) 1:5,000.

Generation of ica and mgrA transcriptional fusions with Gfp

To obtain an *ica* and *mgrA* transcriptional fusions, we amplified the *ica* promoter using primers AU59 and AU76 and the *mgrA* promoter using primers PmgrA-fw and Pmgra-rv (**Table S2**) and cloned in pCN52 plasmid (Charpentier *et al.*, 2004), giving pCN52-$P_{ica(BS)}$-gfp and pCN52-P_{mgrA}-gfp plasmids respectively. Plasmids were transformed in *S. aureus* wild-type strains and *arl* mutants. To analyze *ica* and *mgrA* expression, total protein extracts were recovered and analyzed by SDS-PAGE and Western blot Gfp was detected using anti-GFP (Living Color® A.v. Monoclonal Antibody (JL-8), Clontech) antibodies diluted 1:2,500 in 0.1% PBS-Tween 5% skim-milk. Peroxidase-conjugated goat anti-mouse Immunoglobulin G (Invitrogen) diluted 1:5000 in 0.1% PBS-Tween 5% skim-milk were used as secondary antibodies.

RNA extraction and Northern-blot

Staphylococcus aureus 132 and *arl* strains were grown in 10 ml of TSB-NaCl at 37 °C under static conditions for 5 h. RNA extraction was performed as described

previously (Lasa *et al.*, 2011). Northern blots were performed as described (Ruiz de Los Mozos *et al.*, 2013). Briefly, 8 µg of total RNA was separated in precast agarose gels (Sigma). RNAs were blotted onto Nytran membranes (0.2-µm pore size) (Amersham Biosciences), UV cross-linked, prehybridized in ULTRAhyb solution (Ambion) at 65 °C, and labeled with strand-specific riboprobes specific for *icaA, icaC* and *icaR* (**Table S2**). Membranes were washed and autoradiography images were registered at different exposition times according to each gene.

Protein tagging and immunodetection analysis

Transfer of the 3xFlag sequence into IcaC was achieved by recombination using plasmid pMADicaC-3xFlag. To construct pMADicaC-3xFlag the C-terminal region of *icaC* was amplified using primers CFlag1 and CFlag2 (**Tale S2**). The CFlag2 primer contains 66 nt that code for the 3xFlag sequence. Strains containing IcaC-3xFlag were grown in TSB-NaCl or TSBg at 37 °C under static conditions for 5 h. Then, cells were centrifuged and the pellets were resuspended in PBS buffer containing lysostaphin (12.5 µg/ml; Sigma) and DNase I for 2 h. Samples were subjected to electrophoresis on SDS-PAGE 12% Criterion™ TGX Stain-Free™ Precast Gels (BioRad). Proteins were transferred onto nitrocellulose membranes (Hybond Amersham Biosciences) and 3xFLAG fusion proteins were immunodetected by the use of anti-FLAG M2 mAbs conjugated with peroxidase (Sigma).

Affinity blotting of Ebh

For detection of the levels of Ebh protein overnight cultures of the different strains were diluted 1:100 in 50 ml of TSB-glu medium and grown at 37ºC to a mid-log exponential phase (OD = 0.8). Surface-associated proteins were extracted as previously described (Taglialegna *et al.*, 2016). Proteins were subjected to electrophoresis on SDS-PAGE 7.5% TGX Stain-Free™ FastCast™ Acrylamide Kit (BioRad). For immunoblotting, proteins were transferred onto Hybond-ECL nitrocellulose membranes (Amersham Biosciences). Membranes were blocked for 2 hours at room temperature with 5% skimmed milk in PBS with 0.1% Tween 20 and incubated overnight at 4ºC with specific antibodies for the carboxy-terminal region of Ebh diluted 1:1,000. Bound antibodies were detected with peroxidase-conjugated goat anti-rabbit immunoglobulin G antibodies (Invitrogen) 1:5,000.

Murine model of catheter-associated biofilm formation

To examine the role of the staphylococcal TCSs in implant colonization, an *in vivo* murine model of catheter-associated biofilm formation described by Cucarella et al. was used with some modifications (Cucarella *et al.*, 2001). *S. aureus* strains grown overnight at 37ºC on TSA plates were resuspended in PBS to an optical density of OD_{650nm}=0.2 (10^8 CFU/mL). Groups of 5, five weeks old ICR female mice (Charles River Laboratories) were anesthetized with isoflurane (B. Braun). Two 19 mm intravenous catheters (24G; B. Braun) were aseptically implanted into the subcutaneous interscapular of each mouse and inoculated with 100 µl (10^7 CFU) of *S. aureus* strains. After 5 days, animals were opportunely euthanatized by isoflurane inhalation followed by cervical dislocation. Catheters were aseptically removed, placed in a sterile microcentrifuge tube containing 1 ml of PBS, and vortexed at high speed for 3 min. The number of bacteria was determined by plate count.

Ethics statement

All animal studies were reviewed and approved by the Comité de Ética para la Experimentación Animal (CEEA) of the Universidad de Navarra (approved protocol 032-17). Work was carried out at the Centro de Investigación Médica Aplicada building (ES312010000132) under the principles and guidelines described in European Directive 2010/63/EU for the protection of animals used for experimental purposes.

Statistical analysis

Statistical analyses were performed with the GraphPad Prism 5.01 program. The multiple comparison Tukey´s test was used in mice infection assays to find means that are significantly different from each other.

RESULTS

Involvement of the *S. aureus* TCS signaling system in the colonization of subcutaneous catheters

Since the TCS network is essential for *S. aureus* to adapt to new environments, we hypothesized that one or several TCSs might be involved in environmental sensing and activation of genes that code for biofilm-related factors needed for implant colonization. To address this question, we proceeded to systematically analyze the involvement of each TCS in the colonization of subcutaneous foreign catheters (Cucarella *et al.*, 2001; Vergara-Irigaray *et al.*, 2009) (**Figure 1**) by comparing the levels of catheter-associated biofilm formation of the methicillin-resistant *S. aureus* wild type MW2 strain to those of a collection of single MW2 mutants in each TCS (Villanueva *et al.*, 2018). Because differences in growth rates could affect the capacity to colonize the catheters, we first analyzed the growth rate of each mutant under the laboratory growth conditions. The results revealed no significant differences in growth rates between the collection of mutants deficient in TCS and the wildtype strain (**Supplementary Fig. S1**). Results showed that 3 TCS single mutants, namely *arl*, *srr* and *agr*, showed significantly decreased colonization (**Figure 2**). Interestingly, the *arl* mutant showed the greatest deficit in colonization of implanted catheters amongst all single TCS mutants. These results indicated that ArlRS, SrrAB and Agr TCSs are concomitantly needed for biofilm formation in this DRI model, with ArlRS playing a major role in the process.

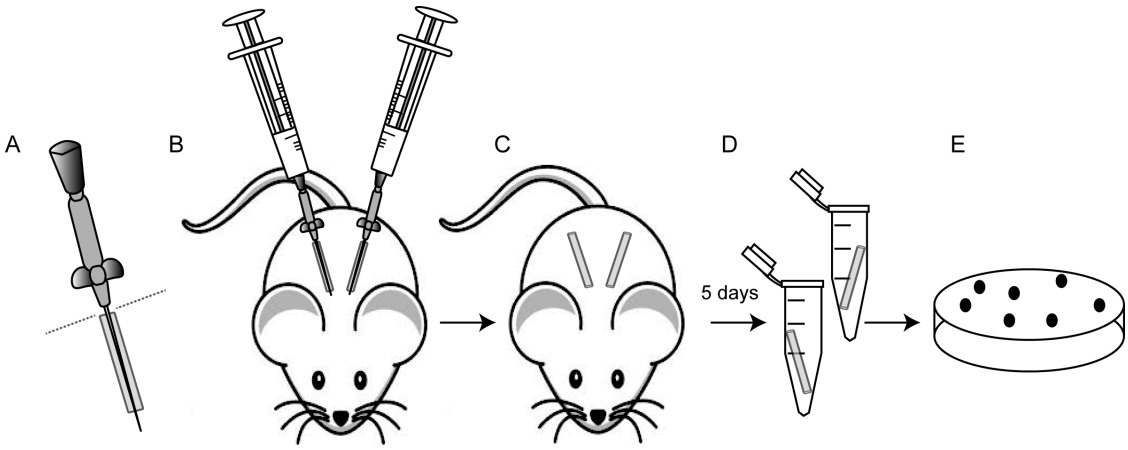

Figure 1. Schematic illustrating the murine model of catheter-associated biofilm formation. (A) ICR female mice were anesthetized by isoflurane and the skin was cleansed with alcohol prior to surgery. The bottom of 19 mm catheters (Introcan Safety 24G; B. Braun) was cut right before insertion (dotted line). **(B)** Then, two catheters were inserted into the subcutaneous interscapular space of each mice and 100 µl of the bacterial suspension containing 10^7 CFU of the strain under study were injected through the Introcan Devices into the catheters. In all the experiments, five mice were used for each strain under study, so that a total of ten catheters were inoculated with each strain. **(C)** Introcan Devices were carefully pulled out from mice and wounds were closured with the tissue adhesive Histoacryl® (B. Braun) so that catheters remained inside for five days. Note that although closuring the wounds, some catheters were naturally pulled out from mice during the course of experiments. **(D)** Mice were euthanized, catheters were aseptically removed, placed in a sterile microcentrifuge tube containing 1 ml of PBS and vigorously vortexed for 3 min to remove adherent bacteria. **(E)** Bacteria were enumerated by viable plate counts.

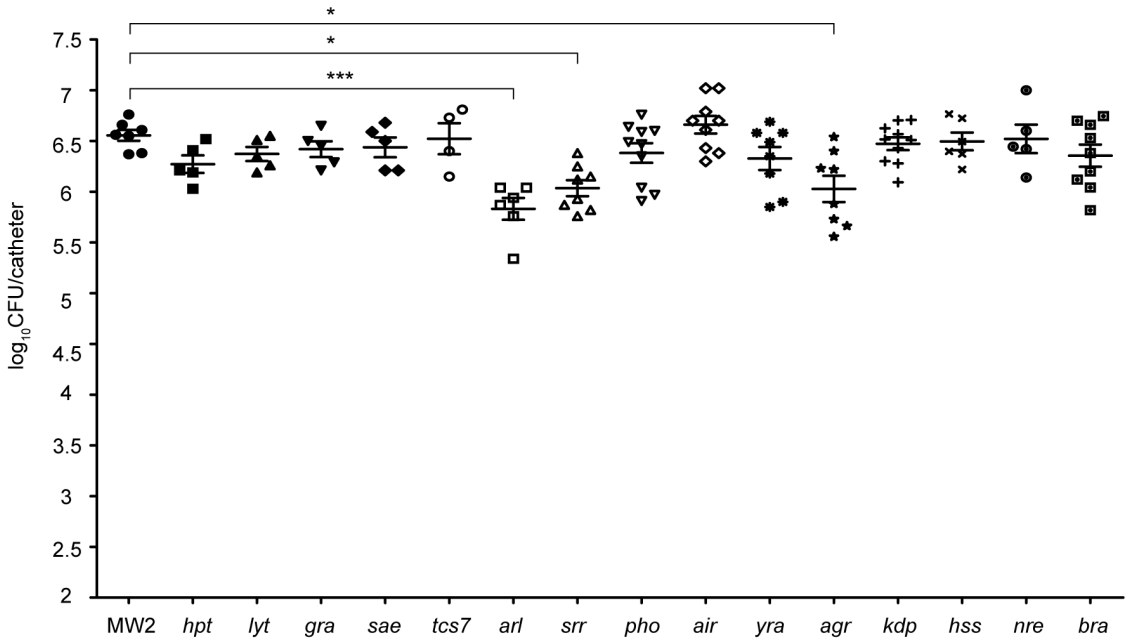

Figure 2. Systematic analysis of the contribution of the TCS signaling system to catheter colonization. Comparison of catheter colonization capacity of the wild type strain (MW2) and single mutants in each non-essential TCS. Bacteria were not detectable in control catheters that had been inoculated with PBS (detection limit 100 CFU/catheter). Note that although a total of ten catheters were inoculated with each strain, a variable number of catheters were recovered in each group due to natural catheter expulsion from mice during the course of the experiment. The plots display values obtained from individual catheters and the mean is represented by horizontal bars. Statistical significance was determined with one-way ANOVA followed by Tukey's multiple comparison test comparing to the WT strain. *P < 0.05; ***P < 0.001..

Synthesis of the PNAG exopolysaccharide is abolished in an *arl* mutant strain

Since the exopolysaccharide PNAG is one of the main components of the *S. aureus* biofilm matrix, we next investigated whether the deficit in catheter colonization shown by the *arl* mutant strain correlated with an altered capacity to produce PNAG. To do so, we determined PNAG production in *arl* by dot-blot, using antibodies anti-PNAG, and compared it to that of the wild type strain and the rest of single MW2 mutants in each TCS. Interestingly, the *arl* mutant strain showed a null capacity to produce PNAG. In contrast, the rest of the mutants displayed a similar, slightly higher or slightly lower PNAG production capacity than the wild type strain (**Figure 3A**). To extend the observed PNAG deficient phenotype of the *arl* mutant to other strain backgrounds, we deleted the *arlRS* genes in the genetically unrelated *S. aureus* strains 132 (methicillin-resistant) and ISP479r (methicillin-susceptible). Deficiency in PNAG production was confirmed in both *arl* mutant strains (**Figure 3B**). Moreover, complementation of the *S. aureus* MW2 *arl* mutant with the pCN51 plasmid expressing the *arl* sensor and response regulator genes under the expression of the PCad promoter (p*arlRS*) led to complete restoration of the capacity to produce PNAG (**Figure 3C**). Such restoration was completely dependent on the presence of *icaADBC* genes, since complementation of the *arl* mutant with p*arlRS* in a Δ*icaADBC* background did not result in PNAG production (**Figure 3C**). Altogether, these results showed that the ArlRS TCS is required for PNAG production in *S. aureus* and suggested a link between the incapacity of *arl* to synthesize PNAG and the deficiency shown by this mutant in the *in vivo* model of subcutaneous catheter colonization.

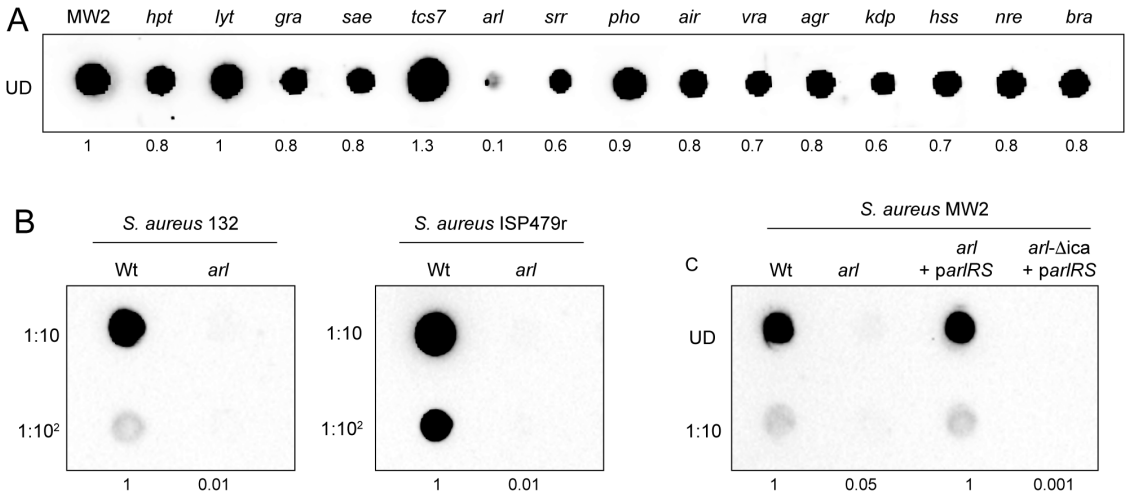

Figure 3. *S. aureus arlRS* mutants do not synthesize PNAG. (A) Dot blot analysis of the PNAG exopolysaccharide synthesized by the wild type MW2 strain and the collection of single mutants in each non-essential TCS. **(B)** Dot blot analysis of PNAG synthesized by the wild type 132 and ISP479r strains and their corresponding *arl* mutants. **(C)** Dot blot analysis of PNAG produced by *S. aureus* MW2, a single *arl* and double *arl*-Δ*ica* mutant strains complemented with plasmid p*arlRS*, which overexpresses the *arlRS* genes under the expression of their own promoter. In all cases, samples were analyzed after 16 h of static incubation, at 37°C, in TSB (MW2 and derivatives), TSB NaCl (132) and TSBg (ISP479r). Serial dilutions (1/10) of the samples were spotted onto nitrocellulose membranes and PNAG production was detected with specific anti PIA/PNAG antibodies. UD; undiluted sample. Numbers below the image show relative dot quantification according to densitometry analysis performed with ImageJ (http://rsbweb.nih.gov/ij/).

Activation of PNAG production by ArlRS is required for in vivo catheter associated biofilm formation

To investigate the possibility that catheter colonization deficiency of the *arl* mutant strain might be due to its failure to produce PNAG, we firstly analyzed catheter colonization capacity of an *arl* mutant in which the *icaADBC* operon was expressed from the chromosome, under the Pcd promoter (*arl*-P_{cd}_ica strain). Restoration of PNAG production in this strain (**Figure 4A**) resulted in colonization levels similar to the wild type strain (**Figure 4B**), indicating that the absence of PNAG in *arl* strain is in fact the reason for its defect in biofilm formation on subcutaneous catheters. Accordingly, a MW2 Δ*icaADBC* mutant, incapable of synthesizing PNAG (**Figure 4A**), showed a significantly reduced capacity to colonize catheters compared with the wild type strain (**Figure 4B**). Additionally, we investigated catheter colonization of *S. aureus* MW2 derivative mutants in surface proteins whose expression is known to be regulated by ArlRS and that are involved in multicellular behavior (Merino *et al.*, 2009; Christner *et al.*, 2010; Crosby *et al.*, 2016). Results showed that neither protein played a role in biofilm formation on subcutaneous catheters (**Supplementary Figure S2**). Overall, these findings indicated that the ArlRS TCS governs the process of *S. aureus* catheter colonization through activation of the synthesis of the PNAG exopolysaccharide, which is fundamental to the formation of a biofilm on the surface of the subcutaneous implanted device.

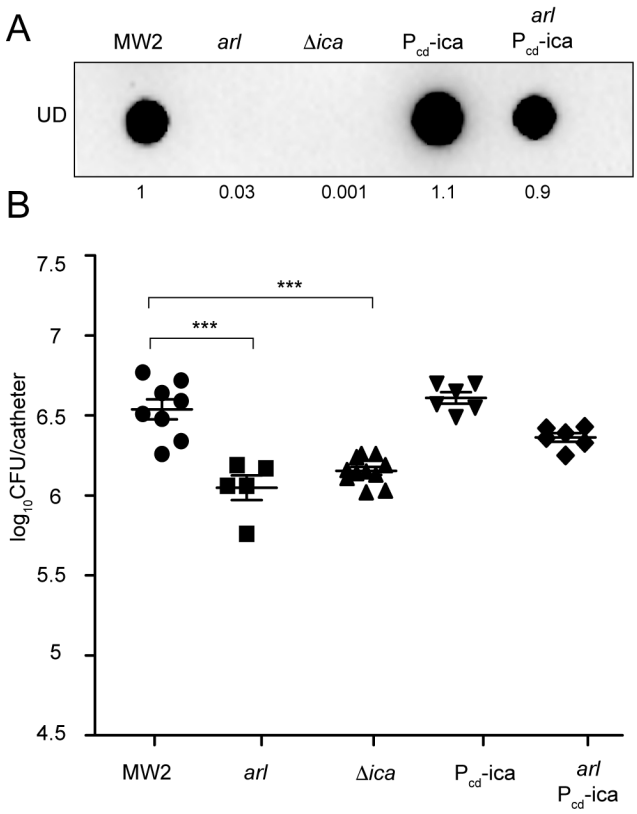

Figure 4. Overexpression of the *icaADBC* operon in an *arl* mutant restores catheter colonization. (A) Dot blot analysis of the PNAG exopolysaccharide synthesized by the wild type MW2, *arl*, Δ*ica* and the wild type and *arl* mutant that overproduce PNAG through the chromosomal expression of the *icaADBC* operon under the Pcad promoter (P_{cd}_ica and *arl* P_{cd}_ica, respectively). Samples were analyzed after 16 h of static incubation, at 37°C, in TSB media. Samples were spotted onto nitrocellulose membranes and PNAG production was detected with specific anti PIA/PNAG antibodies. Numbers below the image show relative dot quantification according to densitometry analysis performed with ImageJ (http://rsbweb.nih.gov/ij/). UD; undiluted sample. **(B)** Comparison of catheter colonization capacity of the strains shown in panel A. Bacteria were not detectable in control catheters that had been inoculated with PBS (detection limit 100 CFU/catheter). Note that although a total of ten catheters were inoculated with each strain, a variable number of catheters were recovered in each group due to natural catheter expulsion from mice during the course of the experiment. The plots display values obtained from individual catheters and the mean is represented by horizontal bars. Statistical significance was determined with one-way ANOVA followed by Tukey's multiple comparison test comparing to the WT strain. ***P < 0.001.

ArlRS regulates *icaADBC* expression at the transcriptional level

It has previously been shown that ArlRS regulates *S. epidermidis* biofilm formation by activating *icaADBC* operon expression, probably through repression of IcaR (Yang Wu, Wang, Xu, Liu, Yu, Lou, Zhu, He, Ben, Hu, Götz, and Qu, 2012a). To analyse if this is the case in *S. aureus*, a sequence upstream of the *ica* operon, including the promoter region of *icaADBC* and the IcaR binding site at the 5`UTR of *ica* genes (-147 to +30), was amplified and cloned into the pCN52 vector (Charpentier *et al.*, 2004), which contains a promoterless *gfpmut2* gene, generating plasmid $P_{ica(BS)}$-gfp. This plasmid was then introduced into *S. aureus* 132 and ISP479r wild type strains and their corresponding *arl* mutants, and expression of the Gfp reporter protein was determined by western-blot. Note that for this analysis, we chose to use such strains instead of *S. aureus* MW2 since as it is shown in **Figure 3**, levels of PNAG production in strains 132 and ISP479r are significantly higher than in strain MW2. Results showed that *arl* mutants expressed much lower levels of Gfp compared to wild-type strains (**Fig. 5A**), indicating that ArlRS activates the expression of the *icaADBC* promoter. Next, we examined, by Northern Blot, whether regulation of *ica* promoter expression by ArlRS leads to altered *icaADBC* mRNA levels, using two different riboprobes specific for *icaA* or *icaC* mRNA. Results showed a strong reduction in the levels of *icaA* and *icaC* mRNA in the *arl* mutant compared with the wild-type strain (**Fig. 5B**). Also, we investigated whether the decrease in the amount of *icaADBC* mRNA in the *arl* mutant correlated with lower levels of Ica proteins. To do so, we tagged the chromosomal copy of the *icaC* gene with the 3XFlag sequence in the wild type 132 and ISP479r strains, *arl* mutants and *arl* mutants complemented with a plasmid expressing the *arlRS* genes (*arl* p*arlRS*), and then, we analysed IcaC production by Western Blot. As expected, IcaC production was abolished in *arl* mutant strains and restored to wild type levels in *arl* p*arlRS* strains (**Figure 5C**). Finally, considering that in *S. epidermidis*, Wu et al. observed an increase in *icaR* expression in an *arlRS* mutant, we wondered whether in *S. aureus*, the ArlRS TCS activates *icaADBC* operon expression through repression of IcaR. Thus, we determined the levels of *icaR m*RNA in the wild type 132 strain and compared them to the levels occurring in the *arl* mutant and the *arl* mutant complemented with a plasmid expressing the *arlRS* genes. As it is shown in **Figure 5D**, mutation of *arl* led to a significant increase in *icaR* mRNA levels that were lowered to approximately wild type levels when the *arl* mutant was complemented with the *arlRS* genes. Collectively, these findings indicated that in *S. aureus*, ArlRS regulates *icaADBC* expression at the

transcriptional level, at least through downregulation of *icaR* expression, resulting in the activation of PNAG production.

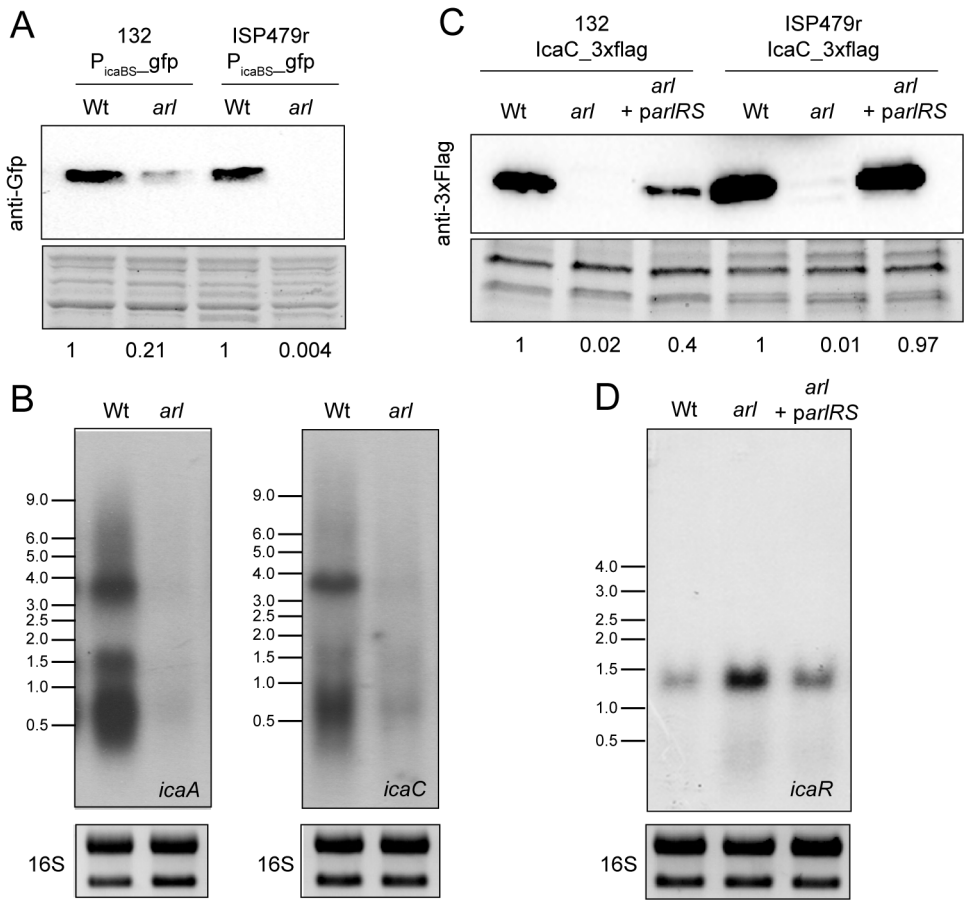

Figure 5. ArlRS transcriptionally activates *icaADBC* operon expression in *S. aureus*. (A) A representative Western blot showing GFP protein levels expressed from *S. aureus* 132 and ISP479r wild type strains and their corresponding *arl* mutants harboring plasmid $P_{ica(BS)}$-gfp. The GFP protein was detected with commercial anti-GFP antibodies. A stain-free gel portion is shown as a loading control. Numbers below the image show relative band quantification according to densitometry analysis performed with ImageJ (http://rsbweb.nih.gov/ij/). **(B)** Representative Northern blots showing *icaA* and *icaC* mRNA of *S. aureus* 132 wild type and *arl* mutant strains grown at 37ºC until exponential phase ($OD_{600\ nm}$ = 0.8). Lower panels show 16S ribosome band stained with RedSafe Nucleic Acid Staining Solution as loading control. **(C)** A representative Western blot showing IcaC protein levels of the wild type 132 and ISP479r strains, *arl* mutants and *arl* mutants complemented with a plasmid expressing the *arlRS* genes (*arl* p*arlRS*). The 3XFlag tagged IcaC protein was detected with commercial anti-3XFlag antibodies. A stain-free gel portion is shown as a loading control. Numbers below the image show relative band quantification according to densitometry analysis performed with ImageJ (http://rsbweb.nih.gov/ij/). **(D)** A representative Northern blot showing *icaR* mRNA of *S. aureus* 132 wild type strain, *arl* mutant and *arl* mutant complemented with a plasmid expressing the *arlRS* genes (*arl* p*arlRS*) grown at 37ºC until exponential phase ($OD_{600\ nm}$ = 0.8). Lower panels show 16S ribosome band stained with RedSafe Nucleic Acid Staining Solution as loading control.

Role of MgrA in the colonization of subcutaneous catheters

It is known that ArlRS and the global regulator MgrA form a regulatory cascade (**Supplementary Figure S3A**) in which MgrA acts downstream of ArlRS to control expression of a number of genes important for virulence, including those for several large surface proteins (Crosby *et al.*, 2016). Also, MgrA seems to regulate PNAG synthesis, since a mutant in *mgrA* has been shown to produce lower levels of PNAG than its corresponding wild type strain (Trotonda *et al.*, 2008). Taken these results into account, we decided to investigate the contribution of MgrA to catheter colonization mediated by the ArlRS TCS. To do so, we firstly constructed a *mgrA* mutant in the MW2 strain and analyzed its capacity to synthesize PNAG by dot blot, using anti PNAG antibodies. The *mgrA* mutant lacked all capacity to produce PNAG (**Figure 6A**). We then compared catheter colonization of the *arl* mutant with that of the *mgrA* mutant and verified that both strains showed a similar deficiency in implant colonization. Importantly, catheter colonization capacity of the *mgrA* mutant was restored when the *icaADBC* operon was expressed from the chromosome, under the Pcad promoter (*mgrA*-P_{cd}_ica strain) (**Figure 6B**), confirming the requirement of PNAG production for implant colonization. Then, we performed epistatic experiments by overexpressing MgrA in the *arlRS* mutant (**Supplementary Figure S3B**) and analyzing PNAG production and colonization capacity of the resulting strain (*arl* P_{cd}_mgrA). Results showed that MgrA overexpression in the *arlRS* background restored neither PNAG production nor implant colonization capacity, indicating that MgrA activity cannot counteract ArlRS absence.

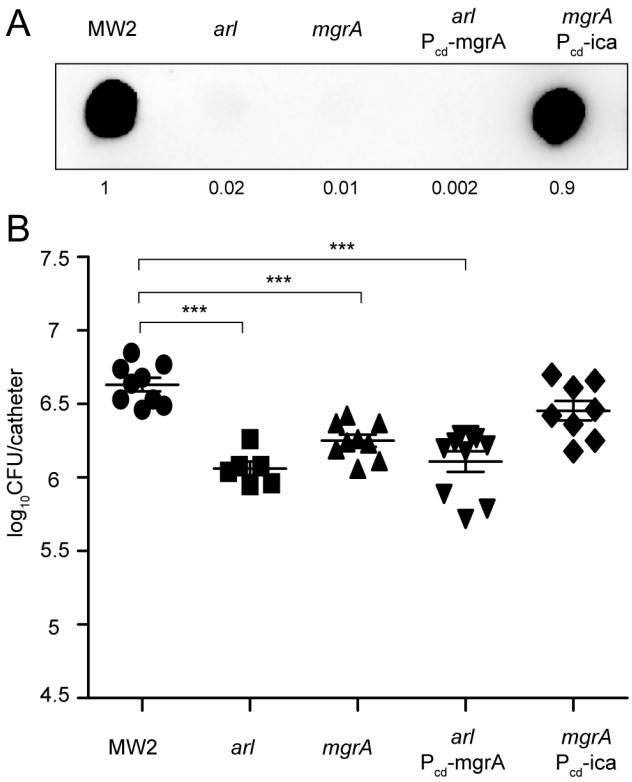

Figure 6. MgrA is unable to restore catheter colonization of an *arl* mutant. (A) Dot blot analysis of the PNAG exopolysaccharide synthesized by the wild type MW2, *arl*, *mgrA*, *arl* that overproduces MgrA through the chromosomal expression of the *mgrA* gene under the Pcad promoter (*arl* P_{cd}_mgrA), and *mgrA* that overproduces PNAG through the chromosomal expression of the *icaADBC* operon under the Pcad promoter (*mgrA* P_{cd}_ica). Samples were analyzed after 16 h of static incubation, at 37°C, in TSB media. Samples were spotted onto nitrocellulose membranes and PNAG production was detected with specific anti PIA/PNAG antibodies. Numbers below the image show relative dot quantification according to densitometry analysis performed with ImageJ (http://rsbweb.nih.gov/ij/). UD; undiluted sample. (**B**) Comparison of catheter colonization capacity of *S. aureus* strains shown in panel A. Bacteria were not detectable in control catheters that had been inoculated with PBS (detection limit 100 CFU/catheter). Note that although a total of ten catheters were inoculated with each strain, a variable number of catheters were recovered in each group due to natural catheter expulsion from mice during the course of the experiment. The plots display values obtained from individual catheters and the mean is represented by horizontal bars. Statistical significance was determined with one-way ANOVA followed by Tukey's multiple comparison test comparing to the WT strain. ***P < 0.001.

DISCUSSION

Many loss-of-function studies have been performed in several pathogenic bacteria to identify the TCSs required for virulence using *in vivo* infection models (Lasaro *et al.*, 2014; Reboul *et al.*, 2014; Cheng *et al.*, 2015; Thomassin *et al.*, 2017). However, to our knowledge, this is the first systematic study conducted to determine the role of each TCS in colonization and survival of *S. aureus* on the surface of an implanted medical device. We used a collection of single mutants in each nonessential TCS of *S. aureus* MW2 and a murine subcutaneous catheter infection model to identify TCSs controlling colonization of the implanted device. It is important to note that none of the single mutants in each TCS show a defect in growth rate when compared to the wild-type strain, at least under the laboratory growth conditions tested (**Supplementary figure 1**). The strongest phenotype in our screening was observed when the *arlRS* TCS was mutated, leading to a greater than 10-fold reduction in the colonization of the catheter. This result was somehow unexpected since other studies have shown that *arlRS* mutants display an increased ability to form a biofilm *in vitro,* due to the release of eDNA (Fournier and Hooper, 2000; Walker *et al.*, 2013) and to the overexpression of SasG, a large surface protein implicated in biofilm formation (Crosby *et al.*, 2016). Furthermore, Toledo-Arana et al also showed that mutation of *arlRS* in *S. aureus* 15981 strain, which is naturally defective in *agr*, promoted biofilm formation in a chemically defined media (HHWm) through overexpression of the protein A surface protein (Toledo-Arana, *et al.*, 2005). In contrast, mutation of *arlRS* in the closely related *S. epidermidis* reduces biofilm formation capacity both *in vitro* and *in viv*o (Yang Wu, Wang, Xu, Liu, Yu, Lou, Zhu, He, Ben, Hu, Götz, and Qu, 2012b; Yang Wu *et al.*, 2014). The molecular mechanism by which ArlRS regulates biofilm formation in *S. epidermidis* has been related with the capacity of ArlR to bind to the promoter region between *icaR* and *icaADBC* and to repress *icaR* expression, which indirectly results in an activation of *icaADBC* expression. In agreement with these results, we showed that ArlRS is involved in the regulation of *icaADBC* expression in *S. aureus*. Deletion of *arlRS* in three genetically unrelated *S. aureus* strains led to a deficiency in PNAG production in TSB media. Transcriptional fusions performed between the *icaADBC* promoter and the *gfp* reporter gene showed that the GFP levels were reduced significantly (about 30%) (**Figure 5A**) in the absence of ArlRS. In addition, *icaC* mRNA levels and consequently, IcaC protein levels, were reduced in the *arl* strain. Thus, we concluded that ArlRS regulates *icaADBC* operon expression at the level of transcription. The control that

ArlRS exerts over *icaADBC* transcription might occur directly or through another regulatory protein(s). Similarly to what has been demonstrated in *S. epidermidis*, we observed that IcaR expression is upregulated in the absence of ArlRS, strongly suggesting that in *S. aureus*, regulation of *icaADBC* transcription by ArlR is mediated through upregulation of IcaR. Further studies are necessary to determine the direct interaction of ArlR with the promoter of the *icaR* gene.

It is believed that the properties provided by the biofilm lifestyle depend on the composition of the biofilm matrix, which can vary not only between strains but also within the same bacterial strain depending on the environmental conditions. *S. aureus* can form biofilms either made of the PNAG exopolysaccharide or composed by surface proteins such as fibronectin binding proteins, FnBPs, protein A, SasG or Bap (Cucarella *et al.*, 2001; Corrigan *et al.*, 2007; Merino *et al.*, 2009; Vergara-Irigaray *et al.*, 2009). Despite the mutation of *arlRS* leads to an increased expression of several surface proteins, including some involved in multicellular behavior, such as SasG, Ebh and Spa (Fournier and Hooper, 2000; Walker *et al.*, 2013; Crosby *et al.*, 2016), in the present study we have shown that none of these proteins seem to play a role in the colonization of implanted catheters. On the contrary, *S. aureus* MW2 required PNAG to form a biofilm on the surface of the subcutaneous catheter. This result was again unexpected since *S. aureus* MW2 is a weak PNAG exopolysaccharide producer under laboratory conditions (Trotonda *et al.*, 2008). This fact warns against the value of *in vitro* PNAG production analysis in order to infer PNAG relevance during infection.

In the present study, we used commercial polyurethane (PU) intravenous catheters. PU is one of the most commonly used material for catheter building due to its high excellent physical properties and good biocompatibility. Analysis of the molecular forces involved in PNAG-mediated adhesion using single-cell force spectroscopy revealed that cationic PNAG binds to anionic surfaces via multivalent electrostatic interactions (Formosa-Dague *et al.*, 2016). During cell-to-cell interactions, it has been proposed that PNAG connects the cells together by electrostatic interaction between its positively charged groups and negatively charged molecules of wall teichoic acids on opposing cells (Gross *et al.*, 2001; Vergara-Irigaray *et al.*, 2008). In the case of cell-implant interaction it seems plausible that PNAG also mediates surface adhesion via electrostatic interactions. On the other hand, adhesion to biological surfaces is often mediated by hydrophobic interactions that are usually the strongest of all long-range non-covalent forces (Briandet *et al.*, 2001). It is known that hydrophobic materials favor bacterial adherence more than hydrophilic ones and consequently modification

of the bacterial surface hydrophobicity can also affect the capacity to colonize the catheter surface (Pavithra and Doble, 2008). We have not addressed whether surface hydrophobicity is modified in the absence of ArlRS and future studies to clarify this possibility are needed. On the other hand, though bacteria can encounter the naked implant surface, we also need to consider that immediately following the implantation of the catheter, a layer of host proteins rapidly adsorbs on its surface, altering the properties of the polymer which may affect bacterial adhesion and biofilm formation. In our experimental model, catheters were infected immediately after implantation without allowing the catheter to be coated with plasma proteins. This model mimics those infection processes that occur during catheter implantation but we cannot exclude that colonization of a catheter surface coated by plasma may involve additional factors.

ArlR activates expression of one of the promoters of the transcriptional regulator MgrA, also called Rat or NorR (Fournier *et al.*, 2000; Ingavale *et al.*, 2003; Crosby *et al.*, 2016). This global regulator modulates the expression of 5-10% of the *S. aureus* genome, and a large part of the ArlRS regulon depends on the expression of MgrA (Luong *et al.*, 2003; Gupta *et al.*, 2015; Crosby *et al.*, 2016). Based on results shown by Trotonda et al. (Trotonda *et al.*, 2008) that indicate that although *S. aureus* mutants in *mgrA* have increased *in vitro* biofilm formation capacity, they produce lower levels of the PNAG exopolysaccharide, we hypothesized that MgrA might be related with the *arlRS* mutant deficiency in catheter colonization. Our results confirmed that an *mgrA* mutant has a decreased PNAG production capacity and also showed that in our catheter infection model, the *mgrA* mutant presents a colonization deficit similar to that of the *arlRS* mutant. Importantly, complementation of both *arlRS* and *mgrA* mutants with the *icaADBC* operon restored implant colonization capacity. However, epistatic experiments revealed that overexpression of MgrA in the *arl* mutant restored neither PNAG production nor implant colonization capacity. These results confirmed the impact that the PNAG exopolysaccharide has for catheter colonization and also indicated that as regards PNAG expression, MgrA activity cannot compensate for ArlRS absence. One plausible explanation for this might be that both regulators regulate *icaADBC* expression at a different level. According to Wu et al (Yang Wu, Wang, Xu, Liu, Yu, Lou, Zhu, He, Ben, Hu, Götz, and Qu, 2012b) and to our results, ArlRS regulates *icaADBC* expression at the transcriptional level through the *icaR* repressor. Instead, MgrA seems to regulate *icaADBC* expression at the posttranscriptional level (Trotonda *et al.*, 2008). These last observations may explain why the *ica* genes have never been

identified in studies carried out to define the MgrA regulon (Luong *et al.*, 2003; Crosby *et al.*, 2016).

Our results also showed that deletion of Agr and SrrAB TCSs cause a slight reduction in the capacity to colonize subcutaneous catheters. Agr controls biofilm architecture and cell dispersion through the regulation of phenol soluble modulins (PSMs) in a *ica*-independent manner (Otto, 2012). PSMs can aggregate and form amyloid fibrils that contribute to stability of the biofilm (Schwartz *et al.*, 2012; Schwartz *et al.*, 2014) Because the levels of the PNAG exopolysaccharide produced *in vitro* were similar between the wild-type and *agr* mutant strains, it is likely that PNAG is not involved in the decreased colonization capacity of the *agr* mutant *in vivo*. On the other hand, SrrAB has been shown to induce biofilm formation under anaerobic conditions through activation of *icaADBC* gene transcription (Ulrich *et al.*, 2007; Youcong Wu *et al.*, 2015) and release of extracellular DNA (Mashruwala *et al.*, 2017). Since implanted catheters are thought to comprise anaerobic microenvironments, it might be possible that the *srrA* mutant has a catheter colonization deficiency because of a defect linked to oxygen sensing.

In summary, we have shown that ArlRS plays a significant role in *S. aureus* implant colonization by affecting the expression of the *icaADBC* operon by directly modifying *icaR* transcription. Although different studies using different genetic strains coincide in showing that ArlRS plays an important role in biofilm mediated infections, it is also noticeable the strong strain and growth media dependency in the observed phenotypes. Thus, additional studies are necessary to determine the precise mechanisms of regulation and the signals to which this two-component system responds.

REFERENCES

Arnaud, M., Chastanet, A., and Débarbouillé, M. (2004) New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. *Appl Environ Microbiol* **70**: 6887–6891.

Aslanidis, C., and de Jong, P.J. (1990) Ligation-independent cloning of PCR products (LIC-PCR). *Nucleic Acids Res* **18**: 6069–6074.

Baba, T., Bae, T., Schneewind, O., Takeuchi, F., and Hiramatsu, K. (2008) Genome sequence of Staphylococcus aureus strain Newman and comparative analysis of staphylococcal genomes: polymorphism and evolution of two major pathogenicity islands. *Journal of Bacteriology* **190**: 300–310.

Briandet, R., Herry, J.M., and Bellon-Fontaine, M.N. (2001) Determination of the van der Waals, electron donor and electron acceptor surface tension components of static Gram-positive microbial biofilms. *Colloids Surf B Biointerfaces* **21**: 299–310.

Charpentier, E., Anton, A.I., Barry, P., Alfonso, B., Fang, Y., and Novick, R.P. (2004) Novel Cassette-Based Shuttle Vector System for Gram-Positive Bacteria. *Appl Environ Microbiol* **70**: 6076–6085.

Cheng, A.T., Ottemann, K.M., and Yildiz, F.H. (2015) Vibrio cholerae Response Regulator VxrB Controls Colonization and Regulates the Type VI Secretion System. *PLoS Pathog* **11**: e1004933.

Cheung, A.L., Bayer, A.S., Zhang, G., Gresham, H., and Xiong, Y.-Q. (2004) Regulation of virulence determinants in vitro and in vivo in Staphylococcus aureus. *FEMS Immunol Med Microbiol* **40**: 1–9.

Christner, M., Franke, G.C., Schommer, N.N., Wendt, U., Wegert, K., Pehle, P., *et al.* (2010) The giant extracellular matrix-binding protein of Staphylococcus epidermidis mediates biofilm accumulation and attachment to fibronectin. *Molecular Microbiology* **75**: 187–207.

Corrigan, R.M., Rigby, D., Handley, P., and Foster, T.J. (2007) The role of Staphylococcus aureus surface protein SasG in adherence and biofilm formation. *Microbiology (Reading, Engl)* **153**: 2435–2446.

Crosby, H.A., Schlievert, P.M., Merriman, J.A., King, J.M., Salgado-Pabón, W., and Horswill, A.R. (2016) The Staphylococcus aureus Global Regulator MgrA Modulates Clumping and Virulence by Controlling Surface Protein Expression. *PLoS Pathog* **12**: e1005604.

Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I., and Penadés, J.R. (2001) Bap, a Staphylococcus aureus surface protein involved in biofilm formation. *Journal of Bacteriology* **183**: 2888–2896.

Ellis, M.W., Schlett, C.D., Millar, E.V., Crawford, K.B., Cui, T., Lanier, J.B., and Tribble, D.R. (2014) Prevalence of nasal colonization and strain concordance in patients with community-associated Staphylococcus aureus skin and soft-tissue infections. *Infect Control Hosp Epidemiol* **35**: 1251–1256.

Formosa-Dague, C., Feuillie, C., Beaussart, A., Derclaye, S., Kucharíková, S., Lasa, I., *et al.* (2016) Sticky Matrix: Adhesion Mechanism of the Staphylococcal Polysaccharide Intercellular Adhesin. *ACS Nano* acsnano.5b07515.

Foster, T.J., Geoghegan, J.A., Ganesh, V.K., and Höök, M. (2014) Adhesion, invasion and evasion: the many functions of the surface proteins of Staphylococcus aureus. *Nat Rev Microbiol* **12**: 49–62.

Fournier, B., and Hooper, D.C. (2000) A new two-component regulatory system involved in adhesion, autolysis, and extracellular proteolytic activity of *Staphylococcus aureus*. *Journal of Bacteriology* **182**: 3955–3964.

Fournier, B., Aras, R., and Hooper, D.C. (2000) Expression of the multidrug resistance transporter NorA from Staphylococcus aureus is modified by a two-component regulatory system. *Journal of Bacteriology* **182**: 664–671.

Gross, M., Cramton, S.E., Gotz, F., and Peschel, A. (2001) Key role of teichoic acid net charge in Staphylococcus aureus colonization of artificial surfaces. *Infection and Immunity* **69**: 3423–3426.

Gupta, R.K., Luong, T.T., and Lee, C.Y. (2015) RNAIII of the Staphylococcus aureus agrsystem activates global regulator MgrA by stabilizing mRNA. *Proc Natl Acad Sci USA* 201509251.

Haag, A.F., and Bagnoli, F. (2016) The Role of Two-Component Signal Transduction Systems in Staphylococcus aureus Virulence Regulation. In *Current Topics in Microbiology and Immunology*. Springer Berlin Heidelberg, Berlin, Heidelberg.

Hogan, S., Stevens, N.T., Humphreys, H., O'Gara, J.P., and O'Neill, E. (2015) Current and future approaches to the prevention and treatment of staphylococcal medical device-related infections. *Curr Pharm Des* **21**: 100–113.

Ingavale, S.S., Van Wamel, W., and Cheung, A.L. (2003) Characterization of RAT, an autolysis regulator in Staphylococcus aureus. *Molecular Microbiology* **48**: 1451–1466.

Kluytmans, J., van Belkum, A., and Verbrugh, H. (1997) Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks. *Clin Microbiol Rev* **10**: 505–520.

Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., *et al.* (2001) Whole genome sequencing of meticillin-resistant Staphylococcus aureus. *Lancet* **357**: 1225–1240.

Lasa, I., Toledo-Arana, A., Dobin, A., Villanueva, M., de los Mozos, I.R., Vergara-Irigaray, M., *et al.* (2011) Genome-wide antisense transcription drives mRNA processing in bacteria. *Proc Natl Acad Sci USA* **108**: 20172–7.

Lasaro, M., Liu, Z., Bishar, R., Kelly, K., Chattopadhyay, S., Paul, S., *et al.* (2014) Escherichia coli isolate for studying colonization of the mouse intestine and its application to two-component signaling knockouts. *Journal of Bacteriology* **196**: 1723–1732.

Lowy, F.D. (1998) Staphylococcus aureus infections. *N Engl J Med* **339**: 520–532.

Luong, T.T., Newell, S.W., and Lee, C.Y. (2003) Mgr, a novel global regulator in Staphylococcus aureus. *Journal of Bacteriology* **185**: 3703–3710.

Maira-Litràn, T., Kropec, A., Abeygunawardana, C., Joyce, J., Mark, G., Goldmann, D.A., and Pier, G.B. (2002) Immunochemical properties of the staphylococcal poly-N-acetylglucosamine surface polysaccharide. *Infection and Immunity* **70**: 4433–4440.

Mashruwala, A.A., Gries, C.M., Scherr, T.D., Kielian, T., and Boyd, J.M. (2017) SaeRS Is Responsive to Cellular Respiratory Status and Regulates Fermentative Biofilm Formation in Staphylococcus aureus. *Infection and Immunity* **85**: e00157–17.

Merino, N., Toledo-Arana, A., Vergara-Irigaray, M., Valle, J., Solano, C., Calvo, E., *et al.* (2009) Protein A-mediated multicellular behavior in Staphylococcus aureus. *Journal of Bacteriology* **191**: 832–843.

Novick, R.P. (2003) Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Molecular Microbiology* **48**: 1429–1449.

O'Gara, J.P. (2007) ica and beyond: biofilm mechanisms and regulation in Staphylococcus epidermidis and Staphylococcus aureus. *FEMS Microbiol Lett* **270**: 179–188.

O'Neill, E., Pozzi, C., Houston, P., Humphreys, H., Robinson, D.A., Loughman, A., *et al.* (2008) A novel Staphylococcus aureus biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. *Journal of Bacteriology* **190**: 3835–3850.

Otto, M. (2012) Molecular basis of Staphylococcus epidermidis infections. *Semin Immunopathol* **34**: 201–214.

Pavithra, D., and Doble, M. (2008) Biofilm formation, bacterial adhesion and host response on polymeric implants--issues and prevention. *Biomed Mater* **3**: 034003.

Reboul, A., Lemaître, N., Titecat, M., Merchez, M., Deloison, G., Ricard, I., *et al.* (2014) Yersinia pestis requires the 2-component regulatory system OmpR-EnvZ to resist innate immunity during the early and late stages of plague. *J Infect Dis* **210**: 1367–1375.

Rice, K.C., Mann, E.E., Endres, J.L., Weiss, E.C., Cassat, J.E., Smeltzer, M.S., and Bayles, K.W. (2007) The cidA murein hydrolase regulator contributes to DNA release and biofilm development in Staphylococcus aureus. *Proc Natl Acad Sci USA* **104**: 8113–8118.

Ruiz de Los Mozos, I., Vergara-Irigaray, M., Segura, V., Villanueva, M., Bitarte, N., Saramago, M., *et al.* (2013) Base Pairing Interaction between 5“- and 3-”UTRs Controls icaR mRNA Translation in Staphylococcus aureus. *PLoS Genet* **9**: e1004001.

Schenk, S., and Laddaga, R.A. (1992) Improved method for electroporation of Staphylococcus aureus. *FEMS Microbiol Lett* **73**: 133–138.

Schwartz, K., Sekedat, M.D., Syed, A.K., O'Hara, B., Payne, D.E., Lamb, A., and Boles, B.R. (2014) The AgrD N-terminal leader peptide of Staphylococcus aureus has cytolytic and amyloidogenic properties. *Infection and Immunity* **82**: 3837–3844.

Schwartz, K., Syed, A.K., Stephenson, R.E., Rickard, A.H., and Boles, B.R. (2012) Functional amyloids composed of phenol soluble modulins stabilize Staphylococcus aureus biofilms. *PLoS Pathog* **8**: e1002744.

Taglialegna, A., Navarro, S., Ventura, S., Garnett, J.A., Matthews, S., Penadés, J.R., *et al.* (2016) Staphylococcal Bap Proteins Build Amyloid Scaffold Biofilm Matrices in Response to Environmental Signals. *PLoS Pathog* **12**: e1005711.

Thomassin, J.-L., Leclerc, J.-M., Giannakopoulou, N., Zhu, L., Salmon, K., Portt, A., *et al.* (2017) Systematic Analysis of Two-Component Systems in Citrobacter rodentium Reveals Positive and Negative Roles in Virulence. *Infection and Immunity* **85**: e00654–16.

Toledo-Arana, A., Merino, N., Vergara-Irigaray, M., Debarbouille, M., Penadés, J.R., and Lasa, I. (2005) Staphylococcus aureus Develops an Alternative, ica-Independent Biofilm in the Absence of the arlRS Two-Component System. *Journal of Bacteriology* **187**: 5318–5329.

Trotonda, M.P., Trotonda, M.P., Tamber, S., Memmi, G., and Cheung, A. (2008) MgrA represses biofilm formation in Staphylococcus aureus. *Infection and Immunity* **76**: 1–11.

Ulrich, M., Bastian, M., Cramton, S.E., Ziegler, K., Pragman, A.A., Bragonzi, A., *et al.* (2007) The staphylococcal respiratory response regulator SrrAB induces *ica* gene transcription and polysaccharide intercellular adhesin expression, protecting *Staphylococcus aureus* from neutrophil killing under anaerobic growth conditions. *Molecular Microbiology* **65**: 1276–1287.

Valle, J., Toledo-Arana, A., Berasain, C., Ghigo, J.-M., Amorena, B., Penadés, J.R., and Lasa, I. (2003) SarA and not sigmaB is essential for biofilm development by Staphylococcus aureus. *Molecular Microbiology* **48**: 1075–1087.

Vergara-Irigaray, M., Maira-Litràn, T., Merino, N., Pier, G.B., Penadés, J.R., and Lasa, I. (2008) Wall teichoic acids are dispensable for anchoring the PNAG exopolysaccharide to the Staphylococcus aureus cell surface. *Microbiology (Reading, Engl)* **154**: 865–877.

Vergara-Irigaray, M., Valle, J., Merino, N., Latasa, C., García, B., Ruiz de Los Mozos, I., *et al.* (2009) Relevant Role of Fibronectin-Binding Proteins in Staphylococcus aureus Biofilm-Associated Foreign-Body Infections. *Infection and Immunity* **77**: 3978–3991.

Villanueva, M., García, B., Valle, J., Rapún, B., Ruiz de Los Mozos, I., Solano, C., *et al.* (2018) Sensory deprivation in Staphylococcus aureus. *Nat Commun* **9**: 523.

Walker, J.N., Crosby, H.A., Spaulding, A.R., Salgado-Pabón, W., Malone, C.L., Rosenthal, C.B., *et al.* (2013) The Staphylococcus aureus ArlRS two-component system is a novel regulator of agglutination and pathogenesis. *PLoS Pathog* **9**: e1003819.

Wu, Yang, Liu, J., Jiang, J., Hu, J., Xu, T., Wang, J., and Qu, D. (2014) Role of the two-component regulatory system arlRS in ica operon and aap positive but non-biofilm-forming Staphylococcus epidermidis isolates from hospitalized patients. *Microb Pathog* **76**: 89–98.

Wu, Yang, Wang, J., Xu, T., Liu, J., Yu, W., Lou, Q., Zhu, T., He, N., Ben, H., Hu, J., Götz, F., and Qu, D. (2012a) The two-component signal transduction system ArlRS regulates Staphylococcus epidermidis biofilm formation in an ica-dependent manner. *PLoS ONE* **7**: e40041.

Wu, Yang, Wang, J., Xu, T., Liu, J., Yu, W., Lou, Q., Zhu, T., He, N., Ben, H., Hu, J., Götz, F., and Qu, D. (2012b) The Two-Component Signal Transduction System ArlRS Regulates Staphylococcus epidermidis Biofilm Formation in an ica-Dependent Manner. *PLoS ONE* **7**: e40041.

Wu, Youcong, Wu, Y., Zhu, T., Han, H., Liu, H., Xu, T., *et al.* (2015) Staphylococcus epidermidis SrrAB regulates bacterial growth and biofilm formation differently under oxic and microaerobic conditions. *Journal of Bacteriology* **197**: 459–476.

SUPPLEMENTARY MATERIAL

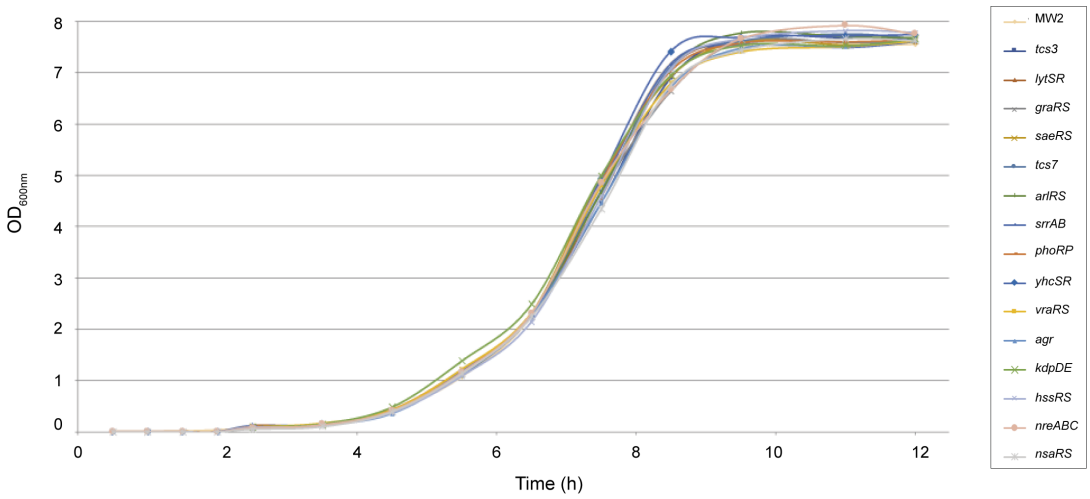

Figure S1. Growth kinetics of MW2 wild-type and their respective mutants in TCSs. Growth curves of *S. aureus* MW2 and the mutants in TCSs grown in TSB-gluc medium at 37ºC. OD 600 nm of the culture was measured every 30 minutes during 12 h.

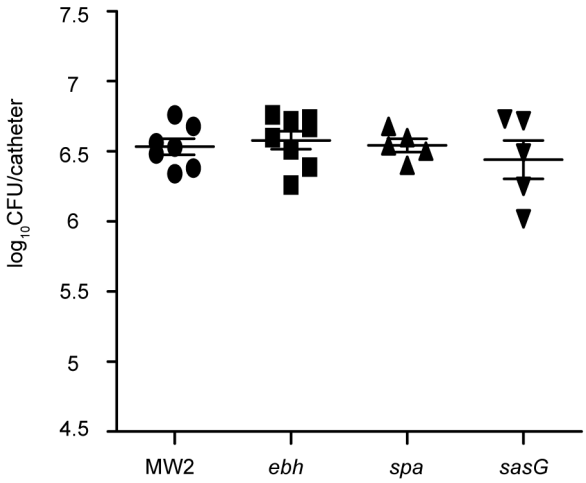

Figure S2. Comparison of catheter colonization capacity of mutants in surface proteins whose expression is regulated by ArlRS. Comparison of catheter colonization capacity of the wild type strain (MW2) and single mutants in *ebh*, *spa* and *sasG*. Bacteria were not detectable in control catheters that had been inoculated with PBS (detection limit 100 CFU/catheter). Note that although a total of ten catheters were contaminated with each strain, a variable number of catheters were recovered in each group due to natural catheter expulsion from mice during the course of the experiment. The plots display values obtained from individual catheters and the mean is represented by horizontal bars. Statistical significance was determined with one-way ANOVA followed by Tukey's multiple comparison test comparing to the WT strain. In all cases, differences were not statistically significant.

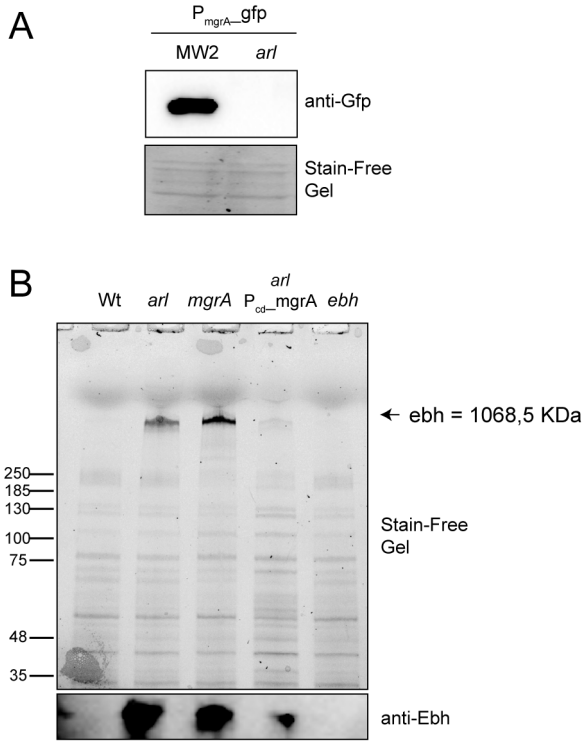

Figure S3. ArlRS controls MgrA expression in *S. aureus* MW2. (A) The P2 promoter of *mgrA* is activated by ArlRS. A representative Western blot showing GFP protein levels expressed from *S. aureus* MW2 wild type and *arl* mutant strains harboring plasmid P_{mgrA}_gfp, which contains the P2 promoter region of *mgrA* fused to a promoterless *gfpmut2* gene. The GFP protein was detected with commercial anti-GFP antibodies. A stain-free gel portion is shown as a loading control. **(B)** Confirmation of the complementation of MgrA expression in Δ*arlRS*- P_{cd}_mgrA strain. It is known that ArlRS represses the expression of the cell wall-associated protein Ebh through MgrA activation. Thus, a deletion of either *arl* or *mgrA* should lead to Ebh overexpression whilst complementation of an *arl* mutant with *mgrA* should result in a repression of Ebh production. Ebh protein levels expressed from *S. aureus* strains MW2 wild type, *arl*, *mgrA* and *arl* that overproduces MgrA through the chromosomal expression of the *mgrA* gene under the Pcad promoter (*arlRS* P_{cd}_mgrA) were analyzed by SDS-PAGE and Western blot, using anti-Ebh antibodies. A mutant in *ebh* was included as a control of no Ebh production. Cell wall extracts were taken at exponential phase ($OD_{600\ nm}$ = 0.8) from strains grown in TSBg at 37º.

CHAPTER III

Biofilm matrix exoproteins induce a protective immune response against Staphylococcus aureus biofilm infection

ABSTRACT

The *Staphylococcus aureus* biofilm mode of growth is associated with several chronic infections that are very difficult to treat due to the recalcitrant nature of biofilms to clearance by antimicrobials. Accordingly, there is an increasing interest in preventing the formation of *S. aureus* biofilms and developing efficient anti-biofilm vaccines. Given the fact that during a biofilm-associated infection, the first primary interface between the host and the bacteria is the self-produced extracellular matrix, in this study, we have analysed the potential of extracellular proteins found in the biofilm matrix to induce a protective immune response against *S. aureus* infections. By using proteomic approaches, we characterized the exoproteomes of exopolysaccharide-based and protein-based biofilm matrices produced by two clinical *S. aureus* strains. Remarkably, results showed that independently of the nature of the biofilm matrix, a common core of secreted proteins is contained in both types of exoproteomes. Intradermal administration of an exoproteome extract of an exopolysaccharide-dependent biofilm induced a humoral immune response and elicited the production of IL-10 and IL-17 in mice. Antibodies against such extract promoted opsonophagocytosis and killing of *S. aureus*. Immunization with the biofilm matrix exoproteome significantly reduced the number of bacterial cells inside a biofilm and on the surrounding tissue, using an *in vivo* model of mesh-associated biofilm infection. Furthermore, immunized mice also showed limited organ colonization by bacteria released from the matrix at the dispersive stage of the biofilm cycle. Altogether, these data illustrate the potential of biofilm matrix exoproteins as a promising candidate multivalent vaccine against *S. aureus* biofilm-associated infections.

INTRODUCTION

Staphylococcus aureus is one of the bacterial species most frequently associated with biofilm-mediated infections. It can be found as a commensal bacterium on the skin, nares and mucosa but in some situations, it can become the source of biofilm-related infections where bacteria grow into multicellular communities attached to a surface and embedded in a self-produced extracellular matrix. *S. aureus* biofilms can occur on host tissues such as heart valves (endocarditis) and bone tissue (osteomyelitis) although they are more frequently related with medical devices (catheters, prostheses, portacaths). Implanted medical devices are easily coated with plasma and extracellular matrix proteins such as fibrinogen and fibronectin (Francois *et al.*, 1998). *S. aureus* has the ability to bind to these components via specific receptors and thus, implants become colonized. After primary attachment to the polymeric surface, bacteria proliferate and accumulate in multilayered clusters surrounded by an extracellular matrix. The added level of bacterial resistance inside a biofilm makes these infections difficult to treat and, as a consequence, in most situations, the device must be surgically removed and replaced (Mader *et al.*, 2000). Bacteria from the biofilm can also propagate through detachment of small or large clumps of cells, or by the release of individual cells allowing bacteria to colonize other surfaces or tissues far from the original infection site. Bloodstream infections originating from device-associated infections account for 11% of all health care–associated infections. An estimation of 250,000 catheter-related bloodstream infections occur in the United States per year, resulting in significant morbidity, mortality, and costs for health care delivery (O'Grady *et al.*, 2002; Maki *et al.*, 2006; Abad and Safdar, 2011). *S. aureus* is frequently associated with such infections, and therefore a great effort is being made in order to prevent and/or obtain effective treatments against this bacterium. Given the fact that bacteria living in a biofilm express a different set of genes than the same free-living bacteria (Beloin *et al.*, 2004; Yao *et al.*, 2005; Waite *et al.*, 2005; Resch *et al.*, 2006; Shemesh *et al.*, 2007), the process of antigen selection for the development of an efficient protection against *S. aureus* infections should also take into consideration those antigens expressed during the biofilm growth.

In this respect, a wide variety of extracellular compounds have been identified as mediators of staphylococcal biofilms such as poly-N-acetyl-glucosamine exopolysaccharide, PNAG (also named PIA), (Heilmann *et al.*, 1996; Mack *et al.*, 1996; McKenney *et al.*, 1999; Cramton *et al.*, 1999; Rupp *et al.*, 2001; Vuong *et al.*, 2004), extracellular DNA (eDNA) (Rice *et al.*, 2007; Izano *et al.*, 2008), and different surface-

associated proteins including the biofilm-associated protein (Bap), fibronectin-binding proteins (FnBPs), SasG and Protein A (Cucarella *et al.*, 2001; Corrigan *et al.*, 2007; O'Neill *et al.*, 2008; Merino *et al.*, 2009; Vergara-Irigaray *et al.*, 2009). Some of these biofilm mediators have been already proposed as vaccine antigens against *S. aureus* infections. Different studies have shown that administration of deacetylated PNAG conjugated with diphtheria toxin as a carrier protein induces an immunological response that protects against *S. aureus* infection (McKenney *et al.*, 1999; Maira-Litràn *et al.*, 2002; Maira-Litran *et al.*, 2005; Pérez *et al.*, 2009). Furthermore, a recent study of Cywes-Benttey *et al.* has shown that PNAG or a structural variant of PNAG is a conserved surface polysaccharide produced by many pathogenic bacteria, fungi and protozoal parasites and has demonstrated that passive immunization with antibodies to PNAG protects mice against both local and systemic infections caused by many of these pathogens (Cywes-Bentley *et al.*, 2013). Protein A and FnBPs have also been evaluated for vaccine development. These antigens generate an immune response that confers partial protection against *S. aureus* challenge using systemic infection models (Zhou *et al.*, 2006; Kim *et al.*, 2010; Kim *et al.*, 2012). However, no evidence has been obtained of the efficiency of these molecules for the protection against biofilm-based infections.

In the last few years, several studies have demonstrated that biofilms harbor multiple cell types, resulting in heterogeneous populations that have followed different developmental pathways (Lazazzera, 2005; An and Parsek, 2007; Vlamakis *et al.*, 2008). In this regard, Brady et al. identified immunogenic cell-wall proteins expressed during a *S. aureus* biofilm infection and demonstrated differing expression patterns for each antigen (Brady *et al.*, 2006; Brady *et al.*, 2007). These authors reasoned that immunization with a monovalent vaccine would likely mean that only a fraction of the biofilm would be targeted and thus, the infection would persist (Harro *et al.*, 2010; Brady *et al.*, 2011). Therefore, they used a quadrivalent vaccine, including four of the identified antigens (glucosaminidase, an ABC transporter lipoprotein, a conserved hypothetical protein, and a conserved lipoprotein), combined with antibiotic therapy and demonstrated a reduced *S. aureus* biofilm formation on infected tibias, using a chronic osteomyelitis model (Brady *et al.*, 2011).

Taking into consideration that the biofilm matrix is the first primary interface between the host and bacteria during a biofilm-associated infection and the relevance of using a multivalent vaccine for the prevention of biofilm-type infections, in this study we aimed at investigating whether an extract containing all proteins secreted into the

biofilm matrix might be a potential polyvalent vaccine candidate that protects against *S. aureus* biofilm related infections. Thus, we have first isolated and identified the exoproteins of both PNAG-dependent and independent biofilm matrices produced by a methicillin sensitive and also a methicillin resistant clinical strain. Notably, exoproteomes were uniform in that they contained a common set of proteins. Immunization with a biofilm matrix exoproteins extract effectively reduced biofilm formation in an *in vivo* model of mesh-associated biofilm infection, which significantly correlated with the production of immunoglobulins (IgG and IgM) antibodies with opsonic activity. Our results also suggested a role for IL-10 and IL-17 cytokines in biofilm matrix exoproteins-mediated protection. Finally, we showed that administration of this multicomponent protein extract reduces organ colonization conducted by bacteria released via detachment from the biofilm.

MATERIAL AND METHODS

Ethics statement

All animal studies were reviewed and approved by the ''Comité de Ética, Experimentación Animal y Bioseguridad'' of the Universidad Pública de Navarra (approved protocol PI-019/12). Work was carried out at the Instituto de Agrobiotecnología building (Idab) under the principles and guidelines described in the ''European Directive 86/609/EEC'' for the protection of animals used for experimental purposes.

Bacterial strains and culture conditions

Staphylococci were cultured on tryptic soy agar or broth at 37°C supplemented with glucose (0.25%) or NaCl (3%) when indicated. Strains used in this study were included in **Table 1**. *S. aureus* 15981, 132 and 12313 were isolated at the Microbiology Department of the Clínica Universidad de Navarra (Pamplona, Spain) (Valle *et al.*, 2003; Vergara-Irigaray *et al.*, 2009). *S. aureus* V329 is a Bap positive strain isolated from a bovine mastitis (Cucarella *et al.*, 2001). *S. aureus* Newman::*bap* is a Newman derivative strain containing a chromosomal copy of the *bap* gene (Arrizubieta *et al.*, 2004). ISP479r is a derivative of ISP479 with a functional *rsbU* gene. As a biofilm negative strain we used *S. aureus* Newman strain (ATCC 25905).

Table 1. Bacterial strains

Strains	Relevant characteristic(s)	Reference or source
S. aureus 15981	MSSA clinical strain. Biofilm positive; PNAG-dependent biofilm matrix	(Valle *et al.*, 2003)
S. aureus 132	MRSA clinical strain. Biofilm positive, able to alternate between a protein-dependent biofilm matrix (grown in TSB-gluc) and a PNAG-dependent biofilm matrix (grown in TSB-NaCl)	(Vergara-Irigaray *et al.*, 2009)
S. aureus ISP479c	MSSA clinical strain. Biofilm positive; PNAG-dependent biofilm matrix	(Pattee, 1981)
S. aureus 12313	MSSA clinical strain. Biofilm positive; PNAG-dependent biofilm matrix	(Vergara-Irigaray *et al.*, 2009)
S. aureus V329	Bovine subclinical mastitis isolate. Biofilm positive; protein-dependent biofilm matrix	(Cucarella *et al.*, 2001)
S. aureus Newman	Strain used in systemic infection models	(Duthie and Lorenz, 1952)

Biofilm formation and protein extracts purification

Biofilm formation under flow conditions was performed using 60-ml microfermenters (Pasteur Institute, Laboratory of Fermentation) with a continuous 40 ml h^{-1} flow of medium and constant aeration with sterile compressed air (0.3 bar) (Ghigo, 2001). Submerged glass slides (spatulas) served as growth substratum. Approximately 10^8 bacteria from an overnight culture of each strain grown in the appropriate medium (*S. aureus* 15981 was grown in TSB-gluc and *S. aureus* 132 was grown either in TSB-gluc or TSB-NaCl) were used to inoculate the microfermenters that were then kept at 37°C for 24 h. The biofilm formed on the spatula was resuspended in 20 ml of PBS (phosphate-buffered saline) and vigorously homogenized by vortexing. The suspension was centrifuged at 4800 g for 30 min at 4ºC. Then, the supernatant was collected, centrifuged again at 4800 g for 30 min at 4ºC and filtered through a 0.45 µm filter (SARSTEDT). Matrix proteins were extracted with trichloroacetic acid 10%. After precipitation, proteins were dissolved in PBS for quantification with the Bicinchoninic Acid Protein Assay kit (SIGMA). The planktonic culture exoproteins extract (PLKE) was obtained as follows. An overnight culture of *S. aureus* 15981 was diluted 1:100 in an Erlenmeyer flask containing 50 ml of TSB-gluc medium and was incubated overnight at 37°C with shaking. The culture was centrifuged at 4800 g. Supernatant was collected and filtered through a 0.45 µm filter (SARSTEDT). Secreted proteins into the supernatant were precipitated by the addition of trichloroacetic acid 10%. Proteins extracts were dissolved in PBS for quantification with the Bicinchoninic Acid Protein Assay kit (SIGMA). Proteins were resolved using SDS-polyacrylamide gel electrophoresis and stained with Bio-Rad Silver Stain according to the manufacturer ´s recommendations. To obtain the bacterial heat extract, a *S. aureus* 15981 cell suspension containing 10^8 CFU was heat inactivated at 80°C for 1 h (Lawrence *et al.*, 2012).

Protein identification

The extracellular protein extract was subjected to tryptic digestion and analyzed as previously described (Merino *et al.*, 2009). Briefly, the tryptic peptide mixtures were injected onto a strong cationic exchange microprecolumn with a flow rate of 30 µl/min as a first-dimension separation. Peptides were eluted from the column as fractions by injecting salt of ammonium acetate of increasing concentrations. Ammonium salts were removed and peptides were analyzed in a continuous acetonitrile gradient on a C18

reversed-phase self-packing nanocolumn. Peptides were eluted (at flow rate of 300 nl/min) from the reversed-phase nanocolumn to a PicoTip emitter nano-spray needle (New Objective, Woburn, MA) for real-time ionization and peptide fragmentation on an Esquire HCT ion trap mass spectrometer (Bruker-Daltonics, Bremen, Germany). Every 1 s, the instrument cycled through acquisition of a full-scan mass spectrum and one MS/MS spectrum. A 4-Da window (precursor m/z ±2), an MS/MS fragmentation amplitude of 0.80 V, and a dynamic exclusion time of 0.30 min were used for peptide fragmentation. 2DnLC was automatically performed on an advanced microcolumn-switching device (Switchos; LC Packings) coupled to an auto-sampler (Famos; LC Packings) and a nano-gradient generator (Ultimate nano- HPLC; LC Packings). The software Hystar 2.3 was used to control the whole analytical process. MS/MS spectra were batch processed by using DataAnalysis 5.1 SR1 and MS BioTools 2.0 software packages and searched against the *S. aureus* protein databases using Mascot software (Matrix Science, London, United Kingdom). The criteria for confirming highly confident protein identification was set at obtaining a MASCOT total protein score ≥ 50 and at least one peptide e-value of ≤ 0.05.

RNA extraction

For planktonic growth conditions, an overnight culture of *S. aureus* 15981 was diluted 1:100 in an Erlenmeyer flask containing 50 ml of TSBgluc medium and was incubated to OD_{600}=0.8 at 37°C with shaking. For biofilm growth conditions, microfermentors where inoculated as described above and incubated at 37ºC for 6 h. Biofilm-grown and planktonically grown cells were harvested. Total RNA from bacterial pellets was extracted by using a TRIzol reagent method (Lasa *et al.*, 2011). Briefly, bacterial pellets were resuspended into 400 µl of solution A (glucose 10%, Tris 12.5 mM, pH 7.6, EDTA 10 mM), mixed to 60 µl of 0.5M EDTA and transferred into Lysing Matrix B tubes containing 500 µl of acid phenol (Ambion). Cells were mechanically lysed by using the Fastprep apparatus (BIO101) at speed 6.0 during 45 s at 4 °C. After centrifugation the aqueous phase was transferred to 2-ml tubes containing 1 ml of TRIzol and 100 µl of chloroform. Tubes were centrifuged and the aqueous phase was transferred into a 2-ml tube containing 200 µl of chloroform, mixed, and incubated for 5 min at room temperature. Tubes were centrifuged and the aqueous phase containing the RNA was precipitated by addition of 500 µl of isopropanol and incubation for 15 min at room temperature. RNA concentrations were quantified, and RNA qualities were

determined by using Agilent RNA Nano LabChips (Agilent Technologies). RNAs were stored at −80 °C until needed.

cDNA labeling and DNA microarray hybridization

Ten µg RNAs were reverse transcribed using SuperScript II reverse transcriptase (Invitrogen Life Technologies). cDNA was digested by DNase I (PIERCE) in 10X DNAse I buffer (USB-Affymetrix) and the size of digestion products was analyzed in the Agilent Bioanalyser 2100 using RNA Nano LabChips to ensure that the fragmentation resulted in a majority of products in the range of 50 to 200 base-pairs. The fragmented cDNA were then biotinylated using terminal deoxynucleotidyl transferase (Promega) and the GeneChip DNA labeling reagent (Affymetrix) following the manufacturer's recommendations. Biotinylated cDNA (5 microgram per array) were hybridized to custom *S. aureus* tiling microarrays designed as described (Segura *et al.*, 2012) (ArrayExpress accession: A-AFFY-165) and incubated for 16 h according to the Affymetrix protocol in a total volume of 200 µl per hybridization chamber. Following incubation, the arrays were washed and stained in the Fluidics station 450 (Affymetrix) using the protocol n°FS450_0005. Scanning of the arrays was then performed using the GeneChip scanner 3000 (Affymetrix). Intensity signals of each probe cells were computed by the GeneChip operating software (GCOS) and stored in cell intensity files (.CEL extension) before preprocessing and analysis. Microarray data were analyzed using limma package (Gentleman *et al.*, 2005). Raw data are available under ArrayExpress accession: E-MEXP-3924.

Immunization studies

CD1 mice were obtained from Charles River and maintained in the animal facility of the Instituto de Agrobiotecnología, Universidad Pública de Navarra. The biofilm matrix exoprotein extract used for immunization consisted of the exoproteins purified from the biofilm matrix produced by *S. aureus* 15981 strain and was referred as BME. Five-week-old female CD1 mice were injected intradermally with 10 µg of BME diluted in adjuvant (Sigma Adjuvant System®). The control group was treated with PBS and adjuvant. Two weeks later, the vaccinated group received a booster dose of 5 µg of BME, while the control group received PBS and adjuvant. Mice were bled via the retroorbital venous plexus on day 0 (pre-immune serum) and 21 days after the first vaccination (immune serum). Both serum samples were analyzed by ELISA and Western Blot for determination of antibody responses against the BME.

Detection of antibodies in the sera

Serum IgG and IgM expression against BME were quantified by coating 96-well ELISA plates (Nunc Maxisorp, Millipore) with 100 µl/well of a 0.1 µg ml^{-1} BME in carbonate buffer (0.5 M; pH 9.4). Plates were incubated at 4ºC overnight. After incubation, wells were then washed three times with PBS containing 0.1% Tween-20 (PBS-T; pH 7.4) and blocked with blocking buffer (5% nonfat dried milk powder in PBS-T) at room temperature for 1h. After washing three times with PBS-T, 100 µl of pre-immune (negative-control) and immune serum diluted 1:100 in PBS were added to each well and incubated at 37ºC for 2 h. After incubation, wells were washed three times with PBS-T and 100 µl of HRP-conjugated goat anti-mouse IgG and IgM (Thermo Scientific) were added to each well. The plates were incubated for 1 h at 37°C and then washed three times. One hundred µl of ABTS solution (diammonium 2,2'-azinobis[3-ethyl-2,3-dihydrobenzothiazole-6-sulphonate]; Millipore) were added to each well and the absorbance at 405nm was determined on an ELISA reader. Results were reported as the OD_{405} of immune serum/OD_{405} of the control serum (T/C).

Immune response was also determined by Western blot. For that, 5 µg of the BME or a planktonic culture exoproteins extract were resolved using SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and incubated with blocking buffer. Then, the membrane was exposed to pre-immune (negative-control) and immune serum at 4ºC overnight. After washing five times with washing buffer (PBS-T 0.1%), the membrane was incubated with goat anti-mouse IgG and IgM (H+L) secondary antibody HRP conjugate and proteins were detected using SuperSignal® WestPico Chemiluminescent Substrate (Thermo Scientific).

Opsonophagocytic assays

Opsonophagocytosis and killing assay has been previously described in (Shahrooei *et al.*, 2012). Briefly, 1 ml of a planktonic culture of strain *S. aureus* 132 grown overnight was pelleted for 5 min at 12,000 g at 4°C, washed twice with PBS, and subsequently diluted to an OD_{600} of 0.5. Bacteria were pre-incubated with 1% or 10% of immune serum, pre-immune serum or PBS for 1 h at 4ºC. The opsonophagocytosis assay was performed with fresh blood obtained from human healthy volunteers. Fresh whole blood from three volunteers was collected and mixed in tubes containing the anticoagulant heparin and then aliquoted into 1.5-ml microcentrifuge tubes (0.5 ml/tube). After pre-incubation, 10 µl of bacterial suspensions were added to the 1.5-ml

microcentrifuge tubes containing 0.5 ml of fresh blood. Tubes were incubated at room temperature with gentle rocking and, after 30 min, samples were serially diluted and plated onto TSA plates to determine the number of surviving CFU. On the other hand, to analyze the opsonophagocytosis and killing of bacteria that are being part of a biofilm, 0.5x0.5cm polypropylene meshes (Prolene®) were incubated with an 1:100 overnight dilution of a culture of the biofilm forming strain *S. aureus* 132 for 2 hours at 37ºC with shaking. Meshes were then washed with PBS and pre-incubated with 1% or 10% of immune serum, pre-immune serum or PBS for 1 h at 4ºC. After pre-incubation, meshes containing bacteria inside a biofilm were added to the 1.5-ml microcentrifuge tubes containing 0.5 ml of fresh blood. Tubes were incubated at room temperature with gentle rocking and, after 30 min, meshes were removed and gently washed and then placed in 1 ml of PBS and vigorously vortexed. Samples were serially diluted and plated onto TSA plates for enumeration of viable staphylococci. Four independent samples of each treatment were performed. The percent amount of bacterial killing was calculated as [1 - (no. of cfu recovered from the treated samples / no. of cfu recovered from the PBS control samples)] *100.

Cytokines production of splenocytes

Groups of 5 CD1 mice were immunized as established before in the "Immunization Studies" section. One week after the second immunization, mice were sacrificed and their spleens were collected under aseptic conditions. Cells suspensions were prepared by resuspending the spleens in RPMI 1640 medium supplemented with 10 % heat-inactivated fetal bovine serum and 1% Penicillin/Streptomycin and subsequent trituration and filtration through a 70 µm nylon mesh. Red blood cells were lysed using ACK lysing buffer. Splenocytes were counted and dispensed into 24-well plates at a concentration of 2×10^5 cells/well. The cells were restimulated with either 1 µg of BME or with PBS during 24, 48 and 96h. The supernatants were harvested and analyzed for interleukins IL-10, IL-2, IL17A and gamma interferon production using their respective ELISA kit (eBioscience) according to the manufacturer´s instructions.

Vaccination/challenge protocol using an in vivo model of mesh-associated biofilm infection

The vaccination protocol was performed as described in the "Immunization Studies" section using BME, PLKE or 10^8 heat-killed bacteria emulsified in adjuvant for immunization. Groups of 6 CD1 mice were used. One week after the second

immunization, a model of mesh-associated biofilm infection was performed as previously described (Lontra *et al.*, 2010) with the following modifications. Prior to surgical procedure, 0.5x0.5cm polypropylene meshes (Prolene®) were incubated with 0.5 ml of a 1:100 overnight dilution of a culture of the biofilm forming strain *S. aureus* 132 for 1 hour and 15 minutes at 37ºC with shaking. To calculate the initial inoculum, duplicate meshes were placed in 1 ml of PBS and vigorously vortexed. Samples were serially diluted and plated onto TSA plates for enumeration of viable staphylococci. Control and vaccinated CD1 mice were anesthetized by intraperitoneal injection of a ketamine/xylazine mixture. After abdominal epilation and antisepsis of the operative field, the animals were operated. An incision of 1.5 cm in the skin was performed with displacement of the subcutaneous space and opening the peritoneal cavity. Then, a mesh, coated with 10^4 CFU of *S. aureus* strain 132, was fixed at the abdominal wall with one anchor point. Finally, the peritoneal cavity was closed by suture with 6/0 Monosyn®. The animals were put in a warm environment and when awake, they were put back in their cages. After 5 days, all animals were sacrificed. Mesh and surrounding tissue were extracted and placed in 1 ml of PBS and vigorously vortexed. Samples were serially diluted and plated onto TSA plates for enumeration of viable staphylococci.

To analyze the additional protection against bacterial population that propagates through detachment from the biofilm, kidneys and liver from the operated animals or from animals challenged by an intravenous injection of a bacterial suspension containing 10^7 CFU of *S. aureus* Newman, were extracted after 5 days. Viable counts were performed on the homogenates by plating the samples on TSA

RESULTS

Identification of the *S. aureus* biofilm matrix exoproteome

In order to isolate and identify the exoproteins present within the biofilm matrix, the biofilm formed by the clinical strain *S. aureus* 15981 grown in TSB-gluc was isolated (Valle *et al.*, 2003). This strain forms a PNAG dependent biofilm when grown under the conditions tested. Exoproteins present within the PNAG-mediated biofilm matrix of 3 independent samples were purified as described in the materials and methods section. Proteins from these extracts were precipitated and then separated by 1-D SDS-PAGE followed by trypsin digestion and identified by 2DnLC-MS/MS. Only proteins identified in at least two of the three samples were considered for further analysis. Thus, a total of 33 extracellular proteins were detected with a MASCOT score higher than 50 (**Table 2**). Importantly, the proteins identified have been recurrently detected in extracellular proteomes of various *S. aureus* isolates (Ziebandt *et al.*, 2001; Nandakumar *et al.*, 2005; Gatlin *et al.*, 2006; Sibbald *et al.*, 2006; Brady *et al.*, 2007; Pocsfalvi *et al.*, 2008; Glowalla *et al.*, 2009; Muthukrishnan *et al.*, 2011; Enany *et al.*, 2013). More notably, 28 out of the 33 proteins identified in our analysis have also been found in the biofilm exoproteome of *S. aureus* D30 strain, isolated from a persistent nasal carrier (**Table 2**) (Muthukrishnan *et al.*, 2011). These data reliably support the validity of the method used to identify exoproteins of the biofilm matrix.

Specifically, exoproteome analyses revealed the presence in the extracellular biofilm matrix of many proteins involved in pathogenesis such as toxins (leukocidin, EsaA and truncated beta-hemolysin) or immunomodulatory proteins (lipoprotein, immunodominant antigen B, immunodominant antigen A, Protein A, IgG-binding protein, secretory antigen precursor SsaA and SceD). The biofilm matrix also contained a markedly large number of proteins involved in carbohydrate metabolism, namely phosphoglycerate mutase, triosephosphate isomerase, enolase, glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate isomerase, alcohol dehydrogenase, L-lactate dehydrogenase and fructose bisphosphate aldolase. Finally, albeit to a lesser extent, enzymes involved in cell-wall peptidoglycan synthesis (autolysin and N-acetylmuramoyl-L-alanine amidase), DNA metabolism and stress proteins (foldase protein, DNA binding protein II, nuclease and superoxide dismutase) were also encompassed in the biofilm exoproteome.

With the aim of extending the biofilm matrix exoproteome analysis to other *S. aureus* strains we used strain 132, which is an MRSA clinical isolate able to alternate

between a PNAG-independent biofilm matrix mediated by the Fibronectin Binding Proteins (FnBPs) and an exopolysaccharidic PNAG-mediated biofilm depending of whether it is cultured under TSB-gluc or TSB-NaCl growing conditions, respectively (Vergara-Irigaray *et al.*, 2009). *S. aureus* 132 was incubated in microfermentors under these two different conditions that allowed the formation of the two biofilm matrices and subsequently, these were isolated for matrix exoproteins identification. Analysis of the PNAG-mediated biofilm matrix revealed the presence of 24 proteins, 17 of which (71%) had been previously identified in the *S. aureus* 15981 strain exoproteome. On the other hand, analysis of the FnBPs-mediated biofilm matrix led to the identification of 19 proteins, being nearly half of them also present in the exoproteome of the PNAG-mediated matrix, and the other half in the *S. aureus* 15981 strain exoproteome. When we considered the biofilm matrices formed by *S. aureus* 132 under these two experimental conditions as a unit, results showed that almost 80% of the matrix exoproteins were included into the biofilm matrix exoproteome of the *S. aureus* 15981 strain.

In conclusion, we identified the PNAG-dependent and FnBPs-dependent biofilm matrix exoproteomes of a methicillin resistant *S. aureus* isolate and also the PNAG-dependent exoproteome of a methicillin sensitive clinical strain. The results indicated that independently of the nature of the biofilm matrix, a common core of secreted proteins is contained in both types of exoproteomes. The biofilm matrix exoprotein extract used for the rest of the study consisted of the 33 exoproteins identified in the biofilm matrix produced by *S. aureus* 15981 strain and was referred as BME.

Transcriptional analysis of genes coding for biofilm matrix exoproteins

Previous studies with several bacteria have shown that gene expression and protein production differ when bacteria are grown under biofilm conditions in comparison with planktonic growth (Beloin *et al.*, 2004; Waite *et al.*, 2005; Resch *et al.*, 2006; Shemesh *et al.*, 2007). Therefore, we proceeded to investigate whether genes coding for the biofilm matrix exoproteins identified with the proteomic analysis were differentially expressed in biofilm conditions with respect to planktonic growth. Transcriptome analyses revealed that *S. aureus* 15981 cells grown under biofilm conditions expressed a markedly different repertoire of genes in comparison to their planktonic counterparts. In total, we observed that 626 genes were differentially expressed under biofilm growing conditions. From these, 276 genes were expressed in

higher amounts in biofilm cells, whilst 350 genes were down-regulated under biofilm conditions. Then, we focused on expression levels of the genes coding for the BME previously identified and found that expression of more than half of the identified proteins (58%) was up-regulated under biofilm growing conditions (**Table 2**). Importantly, genes encoding for 39% of matrix exoproteins were not differentially expressed under biofilm conditions, indicating that the *S. aureus* biofilm matrix encompasses not only proteins that are specific of the biofilm mode of growth, but also a set of proteins that *S. aureus* expresses at the same level during planktonic growth.

Table 2. Biofilm matrix exoproteomes

GenBank accession no.		Putative Protein	*S. aureus* 15981	*S. aureus* 132-PNAG	*S. aureus* 132-FnBPs	Theoric PI	Theoric Mw	Total score[a]	Coverage %	Ratio Exp[b]
Exoproteins up-regulated under biofilm conditions										
gi15927581[c]	SA1813	Leukocidin	x			9.43	40.43	140.5	4.57	32.8
gi15926283[c]	SA0562	Alcohol dehydrogenase Adh1	x	x		5.34	36.05	113.51	10.12	16.6
gi15923805[c]	SA0746	Nuclease	x	x	x	9.27	25.12	1306.41	25.44	14
gi15926008[c]	SA0295	Lipoprotein	x		x	9.49	33.35	321.51	17.15	7.8
gi15927994[c]	SA2204	Phosphoglyceromutase GpmA	x		x	5.23	26.68	191.13	7.02	7.7
gi15928224[c]	SA2431	Immunodominant antigen B IsaB	x		x	9.67	19.37	1995,27	21.14	7.6
g15925815[c]	SA0107	Protein A	x			5.54	56.44	289.57	13.78	6.9
gi15927579	SA1811	Truncated beta-hemolysin Hlb	x		x	7.68	31.26	463.98	6.57	5.9
gi15926570	SA0841	MAP hypothetical protein	x	x	x	9.28	15.84	2018.96	18.06	5.7
gi15925596[c]	SA2399	Fructose-1,6-bisphosphate aldolase	x	x		4.88	33.04	376.91	10.14	4.1
gi15926551[c]	SA0823	Glucose-6-phosphate isomerase Pgi	x	x		4.83	49.82	69.43	8.8	3
gi15926634	SA0900	Cysteine protease precursor SspB	x		x	5.68	44.52	1662.70	24.68	2.9
gi15926265	SA0544	Hypothetical protein		x	x	5.12	29.39	143.28	5.2	2.8
gi15927415[c]	SA1659	Foldase protein PrsA	x	x		9.01	38.64	105.90	3.44	2.8
gi15926291[c]	SA0570	Hypothetical protein	x		x	9.17	18.59	557.99	23.81	2.7
gi15927996[c]	SA2206	IgG-binding protein SBI	x		x	9.38	50.07	172.37	5.87	2.6
gi15927419[c]	SA1663	Hypothetical protein	x	x		4.33	13.31	227.81	34.21	2.6
gi15925985[c]	SA0272	Type VII secretion protein EsaA	x			6.24	114.78	99.62	1.19	2.5
gi15926635[c]	SA0901	Serine protease SspA	x			5.00	36.97	421.27	12.69	2.3
gi15926639[c]	SA0905	Autolysin Atl	x		x	9.60	136.75	3160.15	24.68	2
Exoproteins non-differentially expressed										
gi15926452[c]	SA0730	Phosphoglycerate mutase Pgm	x			4.74	56.42	495.22	16.23	1.8
gi15926451[c]	SA0729	Triosephosphate isomerase TpiA	x	x	x	4.80	27.29	225.47	24.51	1.8
gi15926453[c]	SA0731	Enolase Eno	x	x		4.55	47.12	468.17	7.83	1.7
gi15923272[c]	SA0271	Hypothetical protein	x			4.61	11.04	2443.79	74.23	1.6
gi15926190[c]	SA0471	Cystein synthase CysK		x		5.37	32.97	243.53	7.74	1.6
gi15926073[c]	SA0359	Putative secreted protease inhibitor		x		5.70	21.27	82.70	6.32	1.5
gi15928230[c]	SA2437	N-acetylmuramoyl-L-alanine amidase	x			5.96	69.25	80.54	2.91	1.4
gi15928076[c]	SA2285	Cell wall surface protein SasG		x		5.35	178.53	73.21	1.93	1.4
gi15926396	SA0674	Sulfatase	x	x	x	9.04	74.4	1308.21	4.64	1.3
gi15927054[c]	SA1305	DNA-binding protein II	x		x	9.52	9.63	676.57	52.22	1.2
gi15926091	SA0375	Inositol-monophosphate dehydrogenase		x		4.49	55.81	52.29	2.25	1.2
gi15927699[c]	SA1927	Fructose-bisphosphate aldolase FbaA	x	x		5.01	30.84	776.38	19.23	1.1
gi15926449[c]	SA0727	Glyceraldehyde-3-phosphate dehydrogenase	x	x	x	4.89	36.28	604.09	19.94	1.1
gi15926679[c]	SA0944	Pyruvate dehydrogenase E1 PdhB		x		4.65	35.24	74.69	10.46	1.1
gi15928148[c]	SA2356	Immunodominant antigen A IsaA	x	x	x	6.11	24.2	424.96	23.18	1
g15927884	SA2097	Hypothetical protein			x	5.77	17.4	65.96	9.2	1
gi15925944[c]	SA0232	L-lactate dehydrogenase LctE	x	x	x	4.95	29.45	98.7	11.04	-1

gi15926229	SA0509	Chaperone protein HchA		x		4.90	32.17	95.91	5.14	-1.1
gi15927133[c]	SA1382	Superoxide dismutase SodA	x	x	x	5.08	22.71	3457.35	31.65	-1.1
gi15927879[c]	SA2093	Secretory antigen precursor SsaA homolog	x	x		8.96	29.33	324.85	22.85	-1.9
Exoproteins down-regulated under biofilm conditions										
gi15927670	SA1898	Similar to SceD precursor	x	x		5.52	24.07	57.82	7.79	-4.2

a MASCOT score obtained by2D-LC-MS/MS analysis

b Ratio of gene expression levels between biofilm and planktonic growth conditions

c Also found in *S. aureus* D30 biofilm exoproteome by (Muthukrishnan *et al.*, 2011)

Biofilm extracellular proteins induce a humoral immune response in mice

In order to investigate whether this multivalent extract might be able to induce a protective immune response against *S. aureus*, we firstly evaluated the antibody response in mice immunized with BME. For that, groups of 8 mice were immunized with BME. Blood and sera samples were obtained at day 0 and 21 post immunization and serum IgG and IgM levels were determined by ELISA. Results showed that immunoglobulin levels were significantly higher in sera from mice immunized with BME than in sera from control mice (**Figure 1A**).

Next, BME were separated in a SDS-PAGE gel (**Figure 1B**) and interrogated with a pool of sera obtained either from immunized or control mice. Results showed that the majority of the biofilm matrix exoproteins were recognized by sera from immunized mice while only a slight cross-reaction, probably caused by the presence of Protein A, was observed when sera pool from control mice was used (**Figure 1B**). Also, because BME contains a group of proteins that are equally expressed under biofilm or planktonic growth conditions, we tested immune and control sera against an extract containing extracellular proteins secreted by *S. aureus* cells grown planktonically (PLKE). As expected, immune serum recognized part of the proteins present in the planktonic extract (**Figure 1C**).

Finally, with the aim of analyzing if antibodies raised against the BME extract recognized the biofilm formed by different *S. aureus* strains we isolated biofilm matrix exoproteins from biofilms formed by several *S. aureus* strains and these were interrogated with immune and control sera. In particular, we tested *S. aureus* 132 strain (PNAG and FnBPs mediated biofilms), V329 and Newman::Bap strains (Bap dependent biofilms) and ISP479 and 12313 strains (PNAG mediated biofilms). As it is shown in **figure 1D**, immune sera against the BME extract recognized many proteins present in all extracts analyzed.

Taken together, these data showed that BME was able to induce a humoral immune response and that many of the proteins present in the extract contributed to this immunogenicity. Also, antibodies generated against BME were capable of targeting a broad range of biofilm matrices, suggesting that this multivalent extract might be effective against a large number of relevant biofilm producing strains.

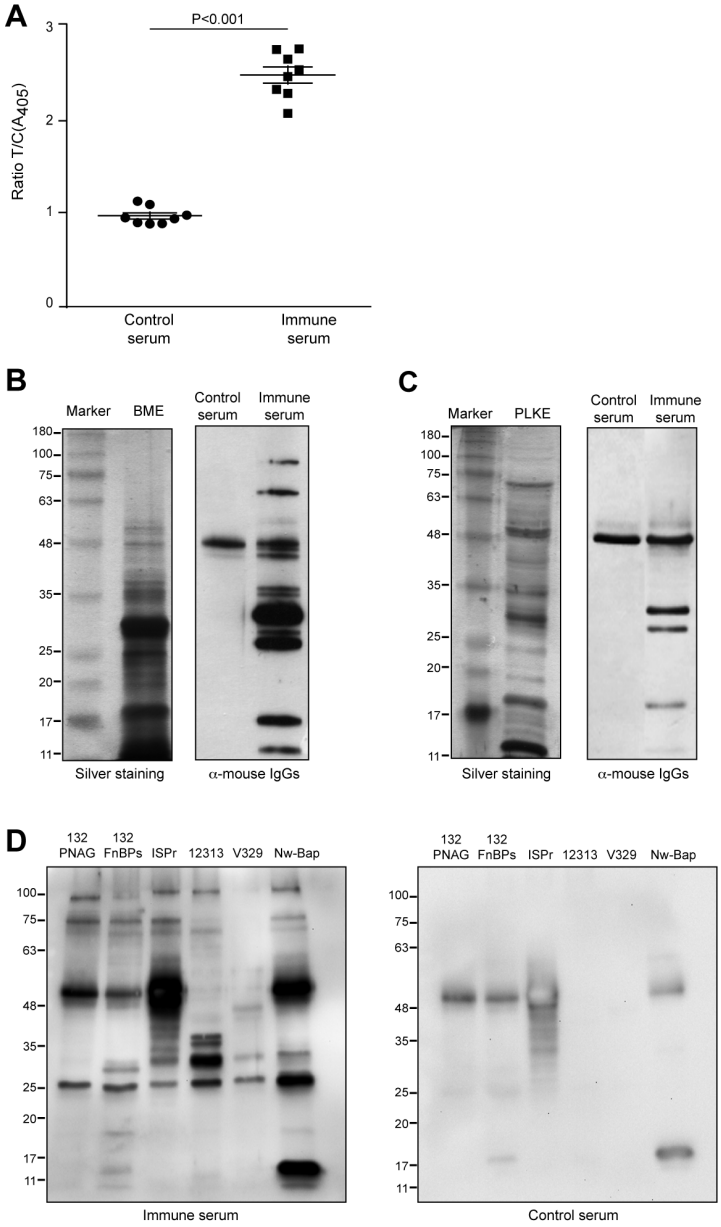

Figure 1. Immunogenicity of the BME extract in mice. Mice (n=8) were immunized twice at an interval of 2 weeks with 10 and 5 µg of the BME extract (treated serum) or with the adjuvant alone (control serum). Sera were collected at times 0 and 1 week after the last immunization. **(A)** IgG titers in response to mice immunization were determined by ELISA. Results were reported as the OD405 of immune serum (treated)/OD405 of the control serum (control) (T/C). The biofilm matrix exoprotein extract (BME) **(B)** and a protein extract (PLKE) coming from the supernatant of a planktonic culture **(C)** were separated on a SDS gel and silver stained. Proteins were transferred to a nitrocellulose membrane by western-blotting and probed with immune or control serum and detected with goat anti-mouse IgG and IgM (H+L) secondary antibody HRP conjugated. **(D)** Western Blot analysis of matrix exoproteins extracts of biofilms formed by different *S. aureus* strains, probed with immune and control serum. *S. aureus* ISP479r (ISPr); *S. aureus* 12313; *S. aureus* V329; *S. aureus* Newman_Bap (Nw-Bap)

Antibodies against BME induce opsonophagocytic killing of *S. aureus*

The presence of IgG and IgM in the immune serum can be correlated with high opsonic activity (Schlageter and Kozel, 1990). Thus, our next objective was to evaluate whether hyperimmune serum obtained against BME promoted opsonophagocytic killing of *S. aureus*. *S. aureus* 132 strain grown under planktonic or biofilm conditions was pre-incubated with preimmune serum, 1% or 10% of BME specific sera or PBS as control. After incubation, bacteria were mixed with whole blood for 30 min (Shahrooei *et al.*, 2012). Staphylococcal killing was monitored by spreading sample aliquots on TSA agar medium followed by colony formation and enumeration. Results showed that antibodies against BME significantly induced opsonophagocytic killing of both planktonic and sessile *S. aureus* cells (**Figure 2**). Additionally, data showed that killing of biofilm *S. aureus* cells was slightly higher than killing of planktonic cells.

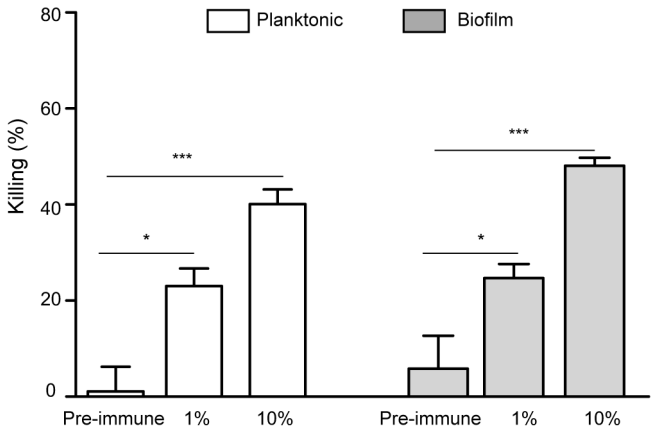

Figure 2. Opsonization with immune serum against BME enhances killing of *S. aureus*. Bacteria grown in planktonic form (white) or attached to polypropylene meshes (grey) were tested for their ability to survive in human blood after preincubation with sterile PBS, preimmune serum, 1% or 10% of immune serum. Surviving bacteria were measured by viable counting. Results are expressed as % of killing calculated as [1 - (no. of cfu recovered from treated samples / no. of cfu recovered from PBS control samples)] *100. Multiple comparisons were performed by one-way analysis of variance combined with the Bonferroni multiple comparison test. GraphPad Instat, version 5).

BME induces the production of IL-10 and IL-17 in *ex-vivo* stimulated splenocytes

We next sought to characterize the cellular response stimulated by BME. For that, cytokine production was examined after *ex-vivo* splenocyte stimulation with BME as described in the materials and methods section. Supernatants of stimulated cells were analyzed for the production of gamma interferon (IFN-γ) and IL-2 (prototype Th1 cytokines), IL-10 (prototype Th2 cytokines) and Th17-associated cytokine IL-17. When production of IL-17 was analyzed over time, a 10 fold increase was observed at the early time of 24 hours post-stimulation, when supernatants of splenocytes coming from mice immunized with the BME extract where compared to supernatants of control mice splenocytes. This difference increased to 50 fold at 96 hours post-stimulation (**Figure 3**). It is important to note that levels of IL-17 over time were barely detectable in supernatants of control mice splenocytes (**Figure 3**). With respect to cytokine IL-10, an approximately 2.5 fold increase was observed at 24 hours post-stimulation that was maintained over time ($P<0.05$) (**Figure 3**). Lastly, mice immunization with BME led to neither stimulation of cytokine IL-2 production nor induction of IFN-γ (**Figure 3**). Taken together, these results showed that immunization with BME induced a cellular response characterized by production of cytokines IL-17 and IL-10.

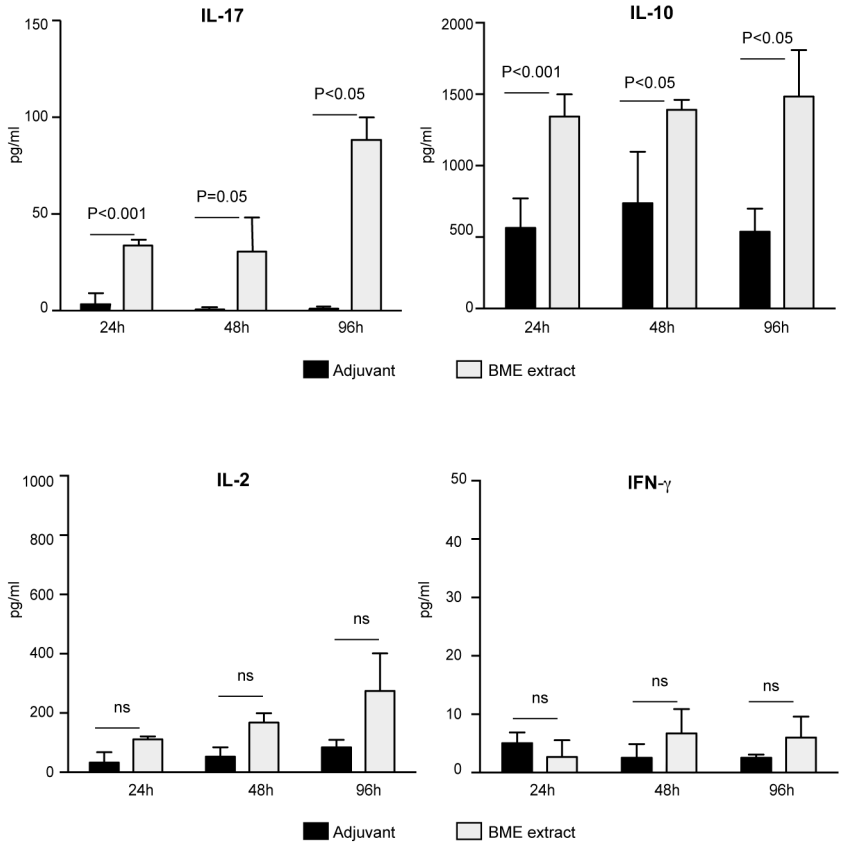

Figure 3. BME-induced production of cytokines in splenocytes. Mice were immunized twice at an interval of 2 weeks with 10 and 5 μg of the BME extract (treated) or with adjuvant alone (control). Three weeks postimmunization, splenocytes were harvested and restimulated for 24h, 48h and 96h with 1 μg of the BME extract. Cell supernatants were harvested and analyzed for IL-2, IL-10, IL17 and IFN-γ production using respective ELISA kits. Results are expressed as pg/ml of each cytokine and are representative of three independent samples. Statistical analysis was carried out using the unpaired Student t test.

Immunization with BME reduced biofilm formation in a mesh-biofilm model

We next hypothesized whether immunization with BME might reduce the number of bacterial cells inside a biofilm formed *in-vivo*. To analyze this hypothesis, we compared the efficiency of BME in a mesh-biofilm model with the protective effect of an extract containing the secreted proteins of *S. aureus* 15981 grown planktonically (PLKE) and also, of a heat extract obtained from *S. aureus* 15981 (HE). Mice were immunized at an interval of two weeks with 10 μg and 5 μg of the BME, PLKE, 10^8 heat-killed bacteria (HE) or with adjuvant alone. After immunization, sera from immunized mice were extracted and were interrogated against the BME proteins. Results showed that sera from mice immunized with PLKE and HE recognized fewer proteins of the BME extract than sera from BME immunized mice (**Figure 4B**).

Seven days after the second immunization, polypropylene meshes coated with 10^4 CFU of the biofilm forming strain *S. aureus* 132 were implanted in the intraperitoneal cavity of immunized and control mice. After five days, all animals were sacrificed and meshes were extracted. When the abdominal cavity of mice was opened, abdominal wall adhesions were observed in all animals. Meshes removed from non-vaccinated mice (control) were more difficult to extract from the abdominal cavity than meshes from vaccinated mice. Also, as it is shown in **figure 4C**, meshes from control mice were surrounded by purulent and necrotic tissue, whilst a healthier and a more vascularized tissue surrounded the meshes coming from immunized mice. When the number of bacteria on meshes was determined, results showed that immunization with BME significantly reduced the number of bacteria attached to the polypropylene meshes ($P \leq 0.05$) (**Figure 4A**). In contrast, immunization with PLKE or HE showed a slight but not statistically significant reduction of the number of bacteria in the mesh-biofilm model (**Figure 4A**).

Finally, we decided to investigate whether BME vaccinated mice were additionally protected against bacterial population that propagates via detachment from the biofilm. To do so, mesh-surrounding tissue, kidneys and liver from BME immunized mice were extracted and bacterial colonization was determined. In contrast to the non-vaccinated group (control), mice immunized with BME presented a significantly reduced number of bacteria in liver and mesh-surrounding tissue ($P \leq 0.05$) (**Figure 4D**). Although there was also a slight reduction in kidney colonization in immunized mice, differences between control and vaccinated mice were not statistically significant ($P=0.06$) (**Figure 4D**).

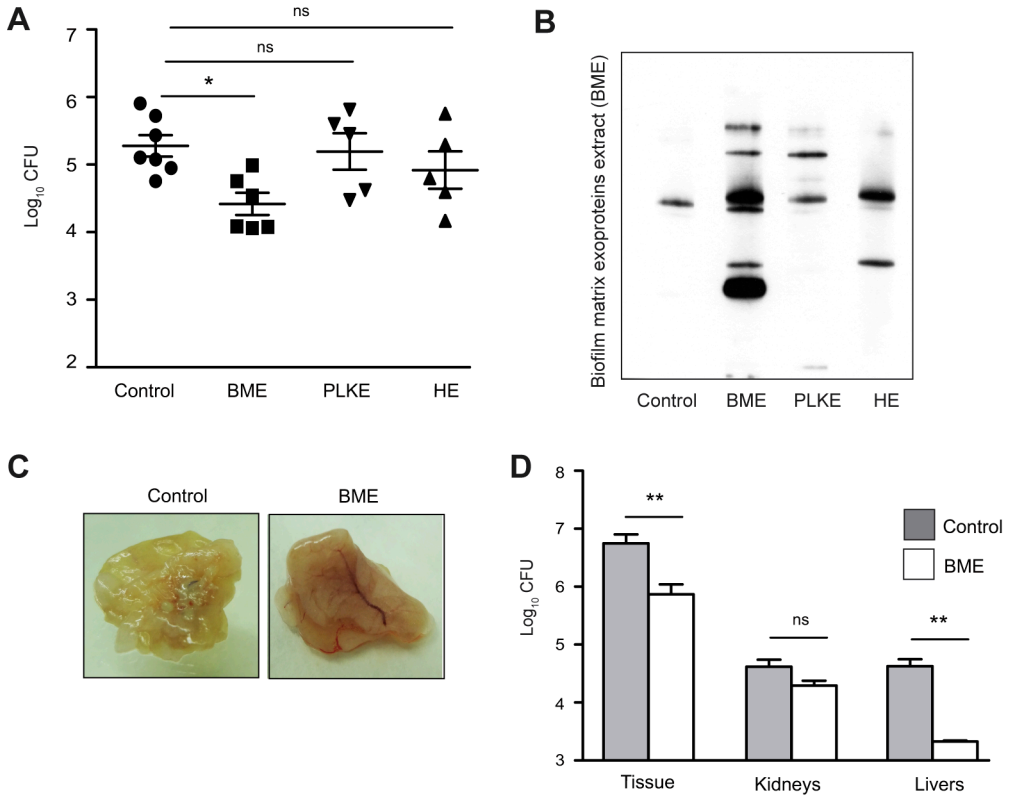

Figure 4. BME extract protects against a biofilm related infection. **(A)** Mice were immunized twice at an interval of 2 weeks with 10 and 5 µg of the BME, PLKE, 10^8 heat-killed bacteria (HE) or with adjuvant alone (control). Polypropylene meshes coated with 10^4 CFU of *S. aureus* strain 132 were fixated at the abdominal wall. After 5 days, animals were sacrificed and meshes were extracted and placed in 1 ml of PBS. Samples were serially diluted and plated onto TSA plates for enumeration of viable staphylococci. Results are representative of six independent mice. Multiple comparisons were performed by one-way analysis of variance combined with the Bonferroni multiple comparison test. (GraphPad Instat, version 5). **(B)** BME proteins were transferred to a nitrocellulose membrane by western-blotting and probed with sera purified from mice immunized with BME, PLKE or HE. **(C)** Images of biofilm infected meshes after 5 days of infection. **(D)** Vaccination with the BME extract also reduces colonization by bacteria that are released from the biofilm. Liver, kidneys and mesh-surrounding tissue from vaccinated and control mice were extracted after five days of insertion of contaminated meshes. Viable staphylococci in the organs and tissue were determined by plate counting.

Reduction in organ colonization in immunized mice might be the consequence of not only reduction of biofilm formation capacity inside the animal and thus, a reduction in the number of released bacteria from the biofilm, but also the efficacy of the immune response against organ colonization by released bacteria. In order to analyze this possibility, we tested whether vaccination with the exoproteins extract might protect against a systemic infection and subsequent organ colonization caused by *S. aureus*. For this, mice were immunized as above and were challenged with a retroorbital injection containing 10^7 cfu/mice of *S. aureus.* Five days after the infection, animals were killed and kidneys and livers were removed. No bacteria were found in the liver of either vaccinated or control mice. Contrary, visual examination of kidneys from non-vaccinated mice showed the presence of abscesses all around the surface of the organs. Much fewer abscesses were detected on kidneys from immunized mice (**Figure 5**). Enumeration of *S. aureus* cells from the organs showed that kidneys of immunized mice were significantly less colonized than kidneys of control mice ($P<0.01$) (**Figure 5**).

From all these results we inferred that immunization with BME significantly reduced biofilm formation in an *in vivo* model of mesh-associated biofilm infection and also moderated organ colonization conducted by bacteria that were released via detachment from the biofilm.

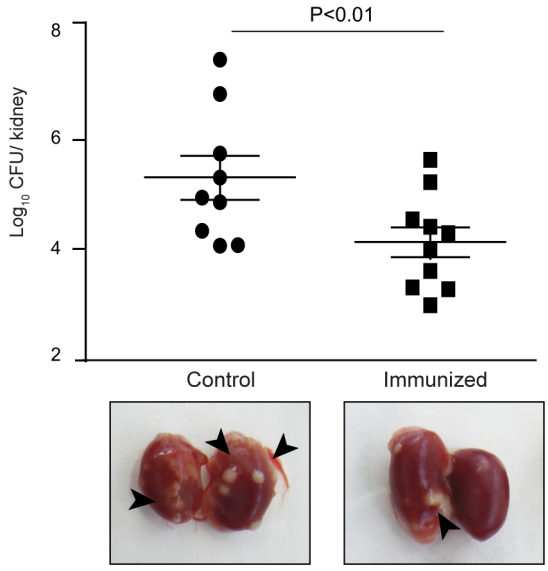

Figure 5. Immunization with the BME extract generates a significant protective immunity against *S. aureus* infection. Vaccinated and control mice were infected with a retrorbital injection containing 10^7 CFU of *S. aureus* Newman. Viable counts were performed on kidney homogenates by plating the samples on TSA. At the bottom, images of abscesses (black arrows) formed in kidneys from control and vaccinated mice are shown.

DISCUSSION

In the last years, *S. aureus* has emerged as one of the most critical nosocomial pathogens. Success of *S. aureus* as a pathogen is the result of different abilities such as the capacity to invade a wide variety of cell types, to secrete a diversity of proteins and toxins and to persist in the host remaining resistant to clearance by the immune system or antibiotics through a biofilm mode of growth. Numerous approaches have been adopted in order to identify staphylococcal surface and cell wall associated proteins as antigenic candidates for a vaccine against *S. aureus* infections (Vytvytska *et al.*, 2002; Etz *et al.*, 2002; Weichhart *et al.*, 2003; Nandakumar *et al.*, 2005; Yang *et al.*, 2006; Gatlin *et al.*, 2006; Clarke *et al.*, 2006; Brady *et al.*, 2006; Glowalla *et al.*, 2009). However, few works have been focused on the selection of antigens that could also protect against biofilm-associated bacteria (McKenney *et al.*, 1999; Maira-Litràn *et al.*, 2002; Maira-Litran *et al.*, 2005; Pérez *et al.*, 2009). This is particularly important because *S. aureus* biofilms play a major role in persistent infections formed on the surface of implanted medical devices and in deep tissues. In this study we have demonstrated that a multicomponent extract containing biofilm matrix exoproteins is able to elicit a protective immune response against *S. aureus* biofilm-mediated infections.

According to Harro et al (Harro *et al.*, 2010), the selection of appropriate antigens effective in preventing the establishment of a biofilm related infection should meet the following criteria: (i) they must be expressed *in vivo* throughout the infection cycle in a large number of genetically unrelated strains; (ii) they must target the entire microbial population of the biofilm; and (iii) they must also induce a protective immune response against planktonic bacteria.

Numerous evidence have demonstrated that *S. aureus* is able to produce polysaccharidic and proteinaceous biofilm matrices (Heilmann *et al.*, 1996; Mack *et al.*, 1996; McKenney *et al.*, 1999; Cramton *et al.*, 1999; Rupp *et al.*, 2001; Vuong *et al.*, 2004). Therefore, potential antigens against *S. aureus* biofilm infections should be expressed by strains that form either type of biofilm matrix. Our results showed that BME extracted from exopolysaccharidic matrices of two unrelated clinical strains (*S. aureus* 15981 and 132) comprised a high number of proteins in common. Moreover, all proteins except one present in the BME isolated from a proteinaceous matrix produced by *S. aureus* 132 were also contained in PNAG-dependent matrices (**Table 2**). Also, it is important to note that 85% of exoproteins encompassed in the BME of *S. aureus* 15981 are identical to the first *S. aureus* biofilm exoproteome identified and produced by the

nasal carrier strain *S. aureus* D30 (Muthukrishnan *et al.*, 2011). Accordingly, here we showed that antibodies raised against an extract coming from a PNAG-dependent biofilm formed by strain 15981 recognized many proteins from biofilms of different nature produced by different *S. aureus* strains (**Figure 1D**). These data might explain why immunization with a BME extract obtained from strain 15981 was effective to protect against a challenge with the clinical relevant MRSA strain *S. aureus* 132 (**Figure 4**) and with *S. aureus* Newman strain (**Figure 5**).

Because individual cells within biofilms can display different protein expression patterns depending on nutrient availability, respiratory conditions or environmental stresses, Harro et al (Harro *et al.*, 2010) proposed that vaccines that only aim at one specific antigen would likely eliminate the section of the biofilm in which the antigen is expressed, whereas, other biofilm areas that do not express the vaccinated antigen will probably persist. Hence, BME extract comprising most exoproteins of the biofilm matrix may ensure that not only different areas of the biofilm but also various cell types present within the biofilm are targeted. It is important to note that vaccination with other multicomponent extracts such as a heat-killed or a PLKE extract, which have been shown to provide protection against *S. aureus* infections (Ekstedt and Yoshida, 1969; Wallemacq *et al.*, 2012; Lawrence *et al.*, 2012), were less efficient than BME to reduce the number of bacteria inside a biofilm, using a mesh associated biofilm infection model. The reason behind the low efficiency of heat-killed and PLKE extracts might be that they probably do not enclose biofilm specific antigens as the BME extract **(Figure 4)**. Additional experimentation will be required to arrive at a detailed picture of the localization of BME proteins into the biofilm structure.

Biofilm formation is a dynamic process that occurs through sequential steps in which the initial attachment of planktonic bacteria to a surface is followed by their subsequent proliferation and accumulation in multilayer cell clusters where bacteria are enclosed in a self-produced polymeric matrix. As biofilm ages, bacterial cells escape from the matrix and return to a planktonic existence, being able to reach other locations in the host. This step represents a potentially important mechanism for the dissemination of bacteria during infection. Our proteomic, transcriptomic and immunological analysis showed that BME extract contains antigens that *S. aureus* produces under both planktonic and biofilm growing conditions **(Table 2)**. As a consequence, sera from BME immunized mice recognized several proteins in the exoproteome extract of planktonic bacteria **(Fig. 1C)**. Accordingly, mice immunized with this extract not only showed a reduction in the number of bacteria inside a *S.*

aureus biofilm but also moderated tissue and organ colonization by bacteria that were released through detachment from the biofilm. Nevertheless, clearance of the infection would likely require an added antimicrobial treatment as it has been already proposed by Brady et al (Brady *et al.*, 2011).

With respect to the immune response mounted after mice immunization with BME, results showed an increase in the production of total immunoglobulins. The primary antibodies function in the protection against *S. aureus* infections is neutralization and opsonization of bacteria for phagocytosis. Although reduction in the number of biofilm bacteria on PS-meshes in the opsonophagocyitc experiment could be due to both neutralization and the opsonic activity of antibodies, we did not observe a significant direct effect of BME-antibodies on *S. aureus* biofilms *in vitro*, in the absence of immune system components (**Supplementary Figure 1**). Hence, BME-antibodies seem to protect against *S. aureus* infections likely through an increase in opsonization. Importantly, these opsonic antibodies may help in the phagocytosis of bacteria inside a biofilm that otherwise would be inaccessible due to the extracellular matrix coating. Although antibodies unquestionably play an important role in the protection against *S. aureus* infections, they may not be decisive for vaccine protective efficacy since animals and humans have enough circulating antibodies to *S. aureus* (Dryla *et al.*, 2005; Clarke *et al.*, 2006; Verkaik *et al.*, 2009). Certain indications show a partial role of these antibodies in protecting humans against staphylococcal infections (Wertheim *et al.*, 2004). However, patients with defects in humoral immunity are not particularly prone to *S. aureus* infections (Proctor, 2012). In this respect, a cellular response mediated by interleukin IL-17 is being considered critical for immunity against this pathogen. It has been shown that vaccination with heat killed *S. aureus* provides protection in systemic infection via staphylococcal lipoproteins that stimulate Th17/IL-17 (Schmaler *et al.*, 2011). Also, IL-17 induction has been shown to be determinant in the clearance of IsdB-immunized mice (Joshi *et al.*, 2012). In biofilm-related infections, IL-17 cytokine production increases during the development of the infection, indicating that infected mice mount a robust Th17 response (Prabhakara *et al.*, 2011; Snowden *et al.*, 2012). Bacteria in biofilm are embedded in an extracellular matrix and are largely protected from phagocytosis by neutrophils and macrophages. The release of inflammatory cytokines by Th17 cells provokes the recruitment and activation of neutrophils and might aid to devitalize the biofilm surface helping to bacterial clearance. In the case of BME extract immunization, it did not only induce a humoral response but also stimulated the production of IL-17 that might help to clear bacteria in the biofilm. In

order to elucidate the role of the induction of IL-17 by BME administration in the efficiency of this multicomponent extract, we have performed a preliminary experiment in which IL-17 cytokine was neutralized by administration of an antibody against IL-17. BME-immunized mice that had been administered the neutralizing antibody to IL-17 showed non-significant reduction in the number of bacteria recovered from biofilm-infected meshes when compared with control BME-immunized mice (**Supplementary Figure 2**). These preliminary results suggest a putative role of IL-17 cytokine in the immune response against a *S. aureus* biofilm related infection. BME immunized mice presented also significantly higher levels of IL-10 compared to non-vaccinated mice. IL-10 cytokine has been shown to protect the host from staphylococcal enterotoxin, endotoxin and septic shock (Howard *et al.*, 1993; Florquin *et al.*, 1994; Haskó *et al.*, 1998). Furthermore, administration of an anti-IL-10 monoclonal antibody to mice inhibits the clearance of *S. aureus,* suggesting that IL-10 might play a beneficial role in host resistance to *S. aureus* systemic infections (Sasaki *et al.*, 2000; Hu *et al.*, 2006). Further studies are needed to explore the role of IL-10 induction by BME administration in the clearance of *S. aureus* biofilm-related infections.

In summary, the work presented here shows that an extract containing biofilm matrix exoproteins induces a protective immune response against a *S. aureus* biofilm related infection and thus reduces colonization and persistence. This is likely because this multicomponent vaccine ties together cell-mediate immunity and a humoral response where opsonic antibodies play a supportive role to eradicate the biofilm infection. In future work, it would be interesting to determine the contribution of each antigen present in the BME extract to its immunogenicity in order to define a particular antigen combination that provides efficient protection against *S. aureus* biofilm infections.

REFERENCES

Abad, C.L., and Safdar, N. (2011) Catheter-related bloodstream infections. *Infectious Disease Special Edition* 14:84.

An, D., and Parsek, M.R. (2007) The promise and peril of transcriptional profiling in biofilm communities. *Current Opinion in Microbiology* **10**: 292–296.

Arrizubieta, M.J., Toledo-Arana, A., Amorena, B., Penadés, J.R., and Lasa, I. (2004) Calcium inhibits bap-dependent multicellular behavior in Staphylococcus aureus. *Journal of Bacteriology* **186**: 7490–7498.

Beloin, C., Valle, J., Latour-Lambert, P., Faure, P., Kzreminski, M., Balestrino, D., *et al.* (2004) Global impact of mature biofilm lifestyle on Escherichia coli K-12 gene expression. *Molecular Microbiology* **51**: 659–674.

Brady, R.A., Leid, J.G., Camper, A.K., Costerton, J.W., and Shirtliff, M.E. (2006) Identification of Staphylococcus aureus proteins recognized by the antibody-mediated immune response to a biofilm infection. *Infection and Immunity* **74**: 3415–3426.

Brady, R.A., Leid, J.G., Kofonow, J., Costerton, J.W., and Shirtliff, M.E. (2007) Immunoglobulins to surface-associated biofilm immunogens provide a novel means of visualization of methicillin-resistant Staphylococcus aureus biofilms. *Appl Environ Microbiol* **73**: 6612–6619.

Brady, R.A., O'May, G.A., Leid, J.G., Prior, M.L., Costerton, J.W., and Shirtliff, M.E. (2011) Resolution of Staphylococcus aureus biofilm infection using vaccination and antibiotic treatment. *Infection and Immunity* **79**: 1797–1803.

Clarke, S.R., Brummell, K.J., Horsburgh, M.J., McDowell, P.W., Mohamad, S.A.S., Stapleton, M.R., *et al.* (2006) Identification of in vivo-expressed antigens of Staphylococcus aureus and their use in vaccinations for protection against nasal carriage. *J Infect Dis* **193**: 1098–1108.

Corrigan, R.M., Rigby, D., Handley, P., and Foster, T.J. (2007) The role of Staphylococcus aureus surface protein SasG in adherence and biofilm formation. *Microbiology (Reading, Engl)* **153**: 2435–2446.

Cramton, S.E., Gerke, C., Schnell, N.F., Nichols, W.W., and Gotz, F. (1999) The intercellular adhesion (ica) locus is present in Staphylococcus aureus and is required for biofilm formation. *Infection and Immunity* **67**: 5427–5433.

Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I., and Penadés, J.R. (2001) Bap, a Staphylococcus aureus surface protein involved in biofilm formation. *Journal of Bacteriology* **183**: 2888–2896.

Cywes-Bentley, C., Skurnik, D., Zaidi, T., Roux, D., Deoliveira, R.B., Garrett, W.S., *et al.* (2013) Antibody to a conserved antigenic target is protective against diverse prokaryotic and eukaryotic pathogens. *Proc Natl Acad Sci USA* **110**: E2209–18.

Dryla, A., Prustomersky, S., Gelbmann, D., Hanner, M., Bettinger, E., Kocsis, B., *et al.* (2005) Comparison of antibody repertoires against Staphylococcus aureus in healthy individuals and in acutely infected patients. *Clin Diagn Lab Immunol* **12**: 387–398.

Duthie, E.S., and Lorenz, L.L. (1952) Staphylococcal coagulase; mode of action and antigenicity. *J Gen Microbiol* **6**: 95–107.

Ekstedt, R.D., and Yoshida, K. (1969) Immunity to staphylococcal infection in mice: effect of living versus killed vaccine, role of circulating antibody, and induction of protection-inducing antigen(s) in vitro. *Journal of Bacteriology* **100**: 745–750.

Enany, S., Yoshida, Y., Magdeldin, S., Bo, X., Zhang, Y., Enany, M., and Yamamoto, T. (2013) Two dimensional electrophoresis of the exo-proteome produced from community acquired methicillin resistant Staphylococcus aureus belonging to clonal complex 80. *Microbiol Res* **168**: 504–511.

Etz, H., Minh, D.B., Henics, T., Dryla, A., Winkler, B., Triska, C., *et al.* (2002) Identification of in vivo expressed vaccine candidate antigens from Staphylococcus aureus. *Proceedings of the National Academy of Sciences* **99**: 6573–6578.

Florquin, S., Amraoui, Z., Abramowicz, D., and Goldman, M. (1994) Systemic release and protective role of IL-10 in staphylococcal enterotoxin B-induced shock in mice. *J Immunol* **153**: 2618–2623.

Francois, P., Vaudaux, P., and Lew, P.D. (1998) Role of plasma and extracellular matrix proteins in the physiopathology of foreign body infections. *Ann Vasc Surg* **12**: 34–40.

Gatlin, C.L., Pieper, R., Huang, S.-T., Mongodin, E., Gebregeorgis, E., Parmar, P.P., *et al.* (2006) Proteomic profiling of cell envelope-associated proteins from Staphylococcus aureus. *Proteomics* **6**: 1530–1549.

Gentleman, R., Carey, V.J., Huber, W., Irizarry, R.A., and Dudoit, S. (2005) *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*. Springer New York, New York, NY.

Ghigo, J.M. (2001) Natural conjugative plasmids induce bacterial biofilm development. *Nature* **412**: 442–445.

Glowalla, E., Tosetti, B., Krönke, M., and Krut, O. (2009) Proteomics-based identification of anchorless cell wall proteins as vaccine candidates against Staphylococcus aureus. *Infection and Immunity* **77**: 2719–2729.

Harro, J.M., Peters, B.M., O'May, G.A., Archer, N., Kerns, P., Prabhakara, R., and Shirtliff, M.E. (2010) Vaccine development in Staphylococcus aureus: taking the biofilm phenotype into consideration. *FEMS Immunol Med Microbiol* **59**: 306–323.

Haskó, G., Virág, L., Egnaczyk, G., Salzman, A.L., and Szabó, C. (1998) The crucial role of IL-10 in the suppression of the immunological response in mice exposed to staphylococcal enterotoxin B. *Eur J Immunol* **28**: 1417–1425.

Heilmann, C., Gerke, C., Perdreau-Remington, F., and Gotz, F. (1996) Characterization of Tn917 insertion mutants of Staphylococcus epidermidis affected in biofilm formation. *Infection and Immunity* **64**: 277–282.

Howard, M., Muchamuel, T., Andrade, S., and Menon, S. (1993) Interleukin 10 protects mice from lethal endotoxemia. *J Exp Med* **177**: 1205–1208.

Hu, D.-L., Omoe, K., Narita, K., Cui, J.-C., Shinagawa, K., and Nakane, A. (2006) Intranasal vaccination with a double mutant of staphylococcal enterotoxin C provides protection against Staphylococcus aureus infection. *Microbes Infect* **8**: 2841–2848.

Izano, E.A., Amarante, M.A., Kher, W.B., and Kaplan, J.B. (2008) Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in Staphylococcus aureus and Staphylococcus epidermidis biofilms. *Appl Environ Microbiol* **74**: 470–476.

Joshi, A., Pancari, G., Cope, L., Bowman, E.P., Cua, D., Proctor, R.A., and McNeely, T. (2012) Immunization with Staphylococcus aureus iron regulated surface determinant B (IsdB) confers protection via Th17/IL17 pathway in a murine sepsis model. *Hum Vaccin Immunother* **8**: 336–346.

Kim, H.K., Cheng, A.G., Kim, H.-Y., Missiakas, D.M., and Schneewind, O. (2010) Nontoxigenic protein A vaccine for methicillin-resistant Staphylococcus aureus infections in mice. *J Exp Med* **207**: 1863–1870.

Kim, H.K., Emolo, C., DeDent, A.C., Falugi, F., Missiakas, D.M., and Schneewind, O. (2012) Protein A-specific monoclonal antibodies and prevention of Staphylococcus aureus disease in mice. *Infection and Immunity* **80**: 3460–3470.

Lasa, I., Toledo-Arana, A., Dobin, A., Villanueva, M., de los Mozos, I.R., Vergara-Irigaray, M., *et al.* (2011) Genome-wide antisense transcription drives mRNA processing in bacteria. *Proc Natl Acad Sci USA* **108**: 20172–7.

Lawrence, P.K., Rokbi, B., Arnaud-Barbe, N., Sutten, E.L., Norimine, J., Lahmers, K.K., and Brown, W.C. (2012) CD4 T cell antigens from Staphylococcus aureus Newman strain identified following immunization with heat-killed bacteria. *Clin Vaccine Immunol* **19**: 477–489.

Lazazzera, B.A. (2005) Lessons from DNA microarray analysis: the gene expression profile of biofilms. *Current Opinion in Microbiology* **8**: 222–227.

Lontra, M.B., Bigolin, A.V., Costa, R.G.D., Grossi, J.V., Scalco, P., Roll, S., and Cavazzola, L.T. (2010) Effectiveness of the combined use of lactic acid film and polypropylene mesh in the formation of intraperitoneal adhesions--an experimental model in rats. *Rev Col Bras Cir* **37**: 364–369.

Mack, D., Fischer, W., Krokotsch, A., Leopold, K., Hartmann, R., Egge, H., and Laufs, R. (1996) The intercellular adhesin involved in biofilm accumulation of Staphylococcus epidermidis is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. *Journal of Bacteriology* **178**: 175–183.

Mader, J.T., Shirtliff, M.E., Bergquist, S., and Calhoun, J.H. (2000) Bone and joint infections in the elderly: practical treatment guidelines. *Drugs Aging* **16**: 67–80.

Maira-Litran, T., Kropec, A., Goldmann, D.A., and Pier, G.B. (2005) Comparative Opsonic and Protective Activities of Staphylococcus aureus Conjugate Vaccines Containing Native or Deacetylated Staphylococcal Poly-N-Acetyl- -(1-6)-Glucosamine. *Infection and Immunity* **73**: 6752–6762.

Maira-Litràn, T., Kropec, A., Abeygunawardana, C., Joyce, J., Mark, G., Goldmann, D.A., and Pier, G.B. (2002) Immunochemical properties of the staphylococcal poly-N-acetylglucosamine surface polysaccharide. *Infection and Immunity* **70**: 4433–4440.

Maki, D.G., Kluger, D.M., and Crnich, C.J. (2006) The risk of bloodstream infection in adults with different intravascular devices: a systematic review of 200 published prospective studies. *Mayo Clin Proc* **81**: 1159–1171.

McKenney, D., Pouliot, K.L., Wang, Y., Murthy, V., Ulrich, M., Döring, G., *et al.* (1999) Broadly protective vaccine for Staphylococcus aureus based on an in vivo-expressed antigen. *Science* **284**: 1523–1527.

Merino, N., Toledo-Arana, A., Vergara-Irigaray, M., Valle, J., Solano, C., Calvo, E., *et al.* (2009) Protein A-mediated multicellular behavior in Staphylococcus aureus. *Journal of Bacteriology* **191**: 832–843.

Muthukrishnan, G., Quinn, G.A., Lamers, R.P., Diaz, C., Cole, A.L., Chen, S., and Cole, A.M. (2011) Exoproteome of Staphylococcus aureus reveals putative determinants of nasal carriage. *J Proteome Res* **10**: 2064–2078.

Nandakumar, R., Nandakumar, M.P., Marten, M.R., and Ross, J.M. (2005) Proteome analysis of membrane and cell wall associated proteins from Staphylococcus aureus. *J Proteome Res* **4**: 250–257.

O'Grady, N.P., Alexander, M., Dellinger, E.P., Gerberding, J.L., Heard, S.O., Maki, D.G., *et al.* (2002) Guidelines for the prevention of intravascular catheter-related infections. *Infect Control Hosp Epidemiol* **23**: 759–769.

O'Neill, E., Pozzi, C., Houston, P., Humphreys, H., Robinson, D.A., Loughman, A., *et al.* (2008) A novel Staphylococcus aureus biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. *Journal of Bacteriology* **190**: 3835–3850.

Pattee, P.A. (1981) Distribution of Tn551 insertion sites responsible for auxotrophy on the Staphylococcus aureus chromosome. *Journal of Bacteriology* **145**: 479–488.

Pérez, M.M., Prenafeta, A., Valle, J., Penades, J., Rota, C., Solano, C., *et al.* (2009) Protection from Staphylococcus aureus mastitis associated with poly-N-acetyl beta-1,6 glucosamine specific antibody production using biofilm-embedded bacteria. *Vaccine* **27**: 2379–2386.

Pocsfalvi, G., Cacace, G., Cuccurullo, M., Serluca, G., Sorrentino, A., Schlosser, G., *et al.* (2008) Proteomic analysis of exoproteins expressed by enterotoxigenic Staphylococcus aureus strains. *Proteomics* **8**: 2462–2476.

Prabhakara, R., Harro, J.M., Leid, J.G., Harris, M., and Shirtliff, M.E. (2011) Murine immune response to a chronic Staphylococcus aureus biofilm infection. *Infection and Immunity* **79**: 1789–1796.

Proctor, R.A. (2012) Is there a future for a Staphylococcus aureus vaccine? *Vaccine* **30**: 2921–2927.

Resch, A., Leicht, S., Saric, M., Pásztor, L., Jakob, A., Götz, F., and Nordheim, A. (2006) Comparative proteome analysis of Staphylococcus aureus biofilm and planktonic cells and correlation with transcriptome profiling. *Proteomics* **6**: 1867–1877.

Rice, K.C., Mann, E.E., Endres, J.L., Weiss, E.C., Cassat, J.E., Smeltzer, M.S., and Bayles, K.W. (2007) The cidA murein hydrolase regulator contributes to DNA release and biofilm development in Staphylococcus aureus. *Proc Natl Acad Sci USA* **104**: 8113–8118.

Rupp, M.E., Fey, P.D., Heilmann, C., and Gotz, F. (2001) Characterization of the importance of Staphylococcus epidermidis autolysin and polysaccharide intercellular adhesin in the pathogenesis of intravascular catheter-associated infection in a rat model. *J Infect Dis* **183**: 1038–1042.

Sasaki, S., Nishikawa, S., Miura, T., Mizuki, M., Yamada, K., Madarame, H., *et al.* (2000) Interleukin-4 and interleukin-10 are involved in host resistance to Staphylococcus aureus infection through regulation of gamma interferon. *Infection and Immunity* **68**: 2424–2430.

Schlageter, A.M., and Kozel, T.R. (1990) Opsonization of Cryptococcus neoformans by a family of isotype-switch variant antibodies specific for the capsular polysaccharide. *Infection and Immunity* **58**: 1914–1918.

Schmaler, M., Jann, N.J., Ferracin, F., and Landmann, R. (2011) T and B cells are not required for clearing Staphylococcus aureus in systemic infection despite a strong TLR2-MyD88-dependent T cell activation. *J Immunol* **186**: 443–452.

Segura, V., Toledo-Arana, A., Uzqueda, M., Lasa, I., and Muñoz-Barrutia, A. (2012) Wavelet-based detection of transcriptional activity on a novel Staphylococcus aureus tiling microarray. *BMC Bioinformatics* **13**: 222.

Shahrooei, M., Hira, V., Khodaparast, L., Khodaparast, L., Stijlemans, B., Kucharíková, S., *et al.* (2012) Vaccination with SesC decreases Staphylococcus epidermidis biofilm formation. *Infection and Immunity* **80**: 3660–3668.

Shemesh, M., Tam, A., and Steinberg, D. (2007) Differential gene expression profiling of Streptococcus mutans cultured under biofilm and planktonic conditions. *Microbiology (Reading, Engl)* **153**: 1307–1317.

Sibbald, M.J.J.B., Ziebandt, A.K., Engelmann, S., Hecker, M., de Jong, A., Harmsen, H.J.M., *et al.* (2006) Mapping the pathways to staphylococcal pathogenesis by comparative secretomics. *Microbiol Mol Biol Rev* **70**: 755–788.

Snowden, J.N., Beaver, M., Smeltzer, M.S., and Kielian, T. (2012) Biofilm-infected intracerebroventricular shunts elicit inflammation within the central nervous system. *Infection and Immunity* **80**: 3206–3214.

Valle, J., Toledo-Arana, A., Berasain, C., Ghigo, J.-M., Amorena, B., Penadés, J.R., and Lasa, I. (2003) SarA and not sigmaB is essential for biofilm development by Staphylococcus aureus. *Molecular Microbiology* **48**: 1075–1087.

Vergara-Irigaray, M., Valle, J., Merino, N., Latasa, C., García, B., Ruiz de Los Mozos, I., *et al.* (2009) Relevant Role of Fibronectin-Binding Proteins in Staphylococcus aureus Biofilm-Associated Foreign-Body Infections. *Infection and Immunity* **77**: 3978–3991.

Verkaik, N.J., de Vogel, C.P., Boelens, H.A., Grumann, D., Hoogenboezem, T., Vink, C., *et al.* (2009) Anti-staphylococcal humoral immune response in persistent nasal carriers and noncarriers of Staphylococcus aureus. *J Infect Dis* **199**: 625–632.

Vlamakis, H., Aguilar, C., Losick, R., and Kolter, R. (2008) Control of cell fate by the formation of an architecturally complex bacterial community. *Genes Dev* **22**: 945–953.

Vuong, C., Kocianova, S., Voyich, J.M., Yao, Y., Fischer, E.R., DeLeo, F.R., and Otto, M. (2004) A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. *J Biol Chem* **279**: 54881–54886.

Vytvytska, O., Nagy, E., Blüggel, M., Meyer, H.E., Kurzbauer, R., Huber, L.A., and Klade, C.S. (2002) Identification of vaccine candidate antigens of Staphylococcus aureus by serological proteome analysis. *Proteomics* **2**: 580–590.

Waite, R.D., Papakonstantinopoulou, A., Littler, E., and Curtis, M.A. (2005) Transcriptome analysis of Pseudomonas aeruginosa growth: comparison of gene expression in planktonic cultures and developing and mature biofilms. *Journal of Bacteriology* **187**: 6571–6576.

Wallemacq, H., Bedoret, D., Pujol, J., Desmet, C., Drion, P.-V., Farnir, F., *et al.* (2012) CD40 triggering induces strong cytotoxic T lymphocyte responses to heat-killed Staphylococcus aureus immunization in mice: a new vaccine strategy for staphylococcal mastitis. *Vaccine* **30**: 2116–2124.

Weichhart, T., Horky, M., Söllner, J., Gangl, S., Henics, T., Nagy, E., *et al.* (2003) Functional selection of vaccine candidate peptides from Staphylococcus aureus whole-genome expression libraries in vitro. *Infection and Immunity* **71**: 4633–4641.

Wertheim, H.F.L., Vos, M.C., Ott, A., van Belkum, A., Voss, A., Kluytmans, J.A.J.W., *et al.* (2004) Risk and outcome of nosocomial Staphylococcus aureus bacteraemia in nasal carriers versus non-carriers. *Lancet* **364**: 703–705.

Yang, G., Gao, Y., Dong, J., Xue, Y., Fan, M., Shen, B., *et al.* (2006) A novel peptide isolated from phage library to substitute a complex system for a vaccine against staphylococci infection. *Vaccine* **24**: 1117–1123.

Yao, Y., Sturdevant, D.E., and Otto, M. (2005) Genomewide analysis of gene expression in Staphylococcus epidermidis biofilms: insights into the pathophysiology of S. epidermidis biofilms and the role of phenol-soluble modulins in formation of biofilms. *J Infect Dis* **191**: 289–298.

Zhou, H., Xiong, Z.-Y., Li, H.-P., Zheng, Y.-L., and Jiang, Y.-Q. (2006) An immunogenicity study of a newly fusion protein Cna-FnBP vaccinated against Staphylococcus aureus infections in a mice model. *Vaccine* **24**: 4830–4837.

Ziebandt, A.K., Weber, H., Rudolph, J., Schmid, R., Hoper, D., Engelmann, S., and Hecker, M. (2001) Extracellular proteins of Staphylococcus aureus and the role of SarA and sigma B. *Proteomics* **1**: 480–493.

SUPPLEMENTARY MATERIAL

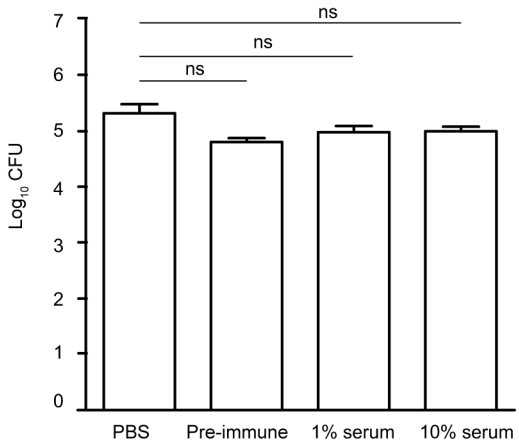

Figure S1: Polypropylene meshes coated with 10^5 CFU of *S. aureus* strain 132 were incubated for 1 h with sterile PBS, preimmune serum, 1% or 10% immune serum. After incubation, meshes were placed in 1 ml of PBS. Samples were serially diluted and plated onto TSA plates for enumeration of viable bacteria. Multiple comparisons were performed by one-way analysis of variance combined with the Bonferroni multiple comparison test (GraphPad Instat, version 5).

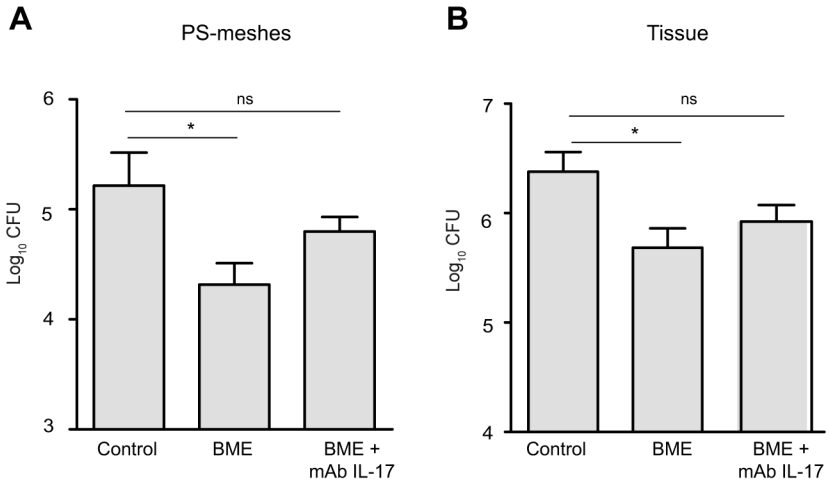

Figure S2: Mice (n=4) were immunized twice at an interval of 2 weeks with 10 and 5 µg of the BME or with adjuvant alone (control). Seven days after the second immunization mice were injected intraperitoneally with 200 µg of neutralizing IL17A antibody (R&D MAB421) (BME + mAb IL17A). After 2 h, polypropylene meshes coated with 10^4 CFU of *S. aureus* strain 132 were fixated at the abdominal wall. Three days after operation, a second dose of 50 µg of neutralizing IL17A antibody were injected in the mice that had been previously treated. After 2 days, animals were sacrificed and meshes (A) and surrounding tissue (B) were extracted and placed in 1 ml of PBS. Samples were serially diluted and plated onto TSA plates for enumeration of viable staphylococci. Results are representative of six independent mice. Multiple comparisons were performed by one-way analysis of variance combined with the Bonferroni multiple comparison test. (GraphPad Instat, version 5).

PERSPECTIVAS DE FUTURO

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La utilización de dispositivos médicos en la medicina moderna está en continuo aumento. A pesar de las múltiples precauciones para evitar infecciones asociadas a los dispositivos, este sigue siendo uno de los mayores problemas asociados a su utilización. Por poner un ejemplo concreto. En nuestra comunidad, se colocaron 1.599 prótesis de rodilla o cadera en el año 2016, de las cuales se infectaron y necesitaron reingreso hospitalario 64 pacientes, lo cual representa de media un 4% de las prótesis implantadas. De aquellas prótesis que han sufrido un primer episodio de infección, se produce un segundo reingreso en 41 pacientes, lo que representa el 64%. Si tenemos en cuenta que cada prótesis de rodilla, cuesta entorno a 6.000 €, simplemente el gasto adicional que ha supuesto a nuestra comunidad las infecciones asociadas a estos implantes ha sido de 1,4 millones de euros. El ratio de infección (4% de prótesis infectadas) varia según el centro hospitalario donde se ha realizado la intervención, pero en su conjunto no es un valor mucho mas elevado que los porcentajes de infección publicados en otros países (Corvec *et al.*, 2012). *S. aureus* se encuentra entre las principales causas de infección de dispositivos médicos y es probablemente la bacteria que mayor alarma genera debido a su virulencia y mala respuesta a los tratamientos antibióticos. Varias razones pueden explicar su protagonismo en este tipo de infecciones: i) se trata de una bacteria ubicua, que coloniza la piel de un gran número de pacientes y personal sanitario, lo que facilita el acceso al material protésico; ii) es una bacteria que muestra una gran capacidad para adherirse a los implantes debido a la gran cantidad de adhesinas que produce la bacteria cuyos ligandos son proteínas presentes en el plasma y en la superficie de los tejidos. Tras la adhesión, la capacidad para formar la matriz del biofilm también es muy amplia ya que es capaz de formar biofilms de naturaleza proteica o polisacarídica; iii) la bacteria produce un gran arsenal de factores de virulencia y toxinas que le ayudan a evadir la acción del sistema inmune y provocar la destrucción del tejido circundante; y iv) muchos aislados son multirresistentes a los antibióticos y el crecimiento en biofilm incrementa, aún mas, esta resistencia.

Aunque el estudio y conocimiento del proceso de formación del biofilm de *S. aureus* ha aumentado significativamente, los esfuerzos realizados para mejorar el tratamiento de las infecciones estafilocócicas asociadas a los dispositivos médicos aún no se han traducido en opciones terapéuticas complementarias a las existentes. En el trabajo realizado en esta tesis hemos explorado nuevas estrategias que podrían

utilizarse para evitar o facilitar el control de las infecciones causadas por los biofilms de *S. aureus*. En esta sección, se destacan reflexiones relacionadas con los resultados obtenidos, así como las estrategias y aspectos que deberían tenerse en cuenta en los futuros estudios dedicados a resolver este problema.

1)

Los resultados obtenidos en el **Capítulo I** muestran que la tecnología DLIP permite modificar las superficies de poliestireno con relieves micro-estructurados que modifican la adhesión de *S. aureus* sobre las superficies.

Hasta el momento los métodos que se utilizan para la fabricación de superficies estructuradas con propiedades antibacterianas incluyen la tecnología de autoensamblado (layer self assembly, LBLSA) y la litografía de superficies. Sin embargo, estas técnicas presentan varias limitaciones; la tecnología LBLSA permite generar estructuras sobre superficies con patrones desordenados y por lo tanto no reproducibles, mientras que la litografía requiere la utilización de máscaras que únicamente pueden ser empleadas sobre superficies planas y de gran tamaño. El desarrollo la tecnología DLIP permite producir relieves definidos en la escala de los micro/nanómetros sobre diversas superficies, a elevadas velocidades de fabricación (50 cm^2/s) y con un coste reducido. En comparación con otros métodos físicos el tratamiento con láser ofrece un enorme potencial para el desarrollo industrial de superficies con relieves micro/nanoestructurados que presenten propiedades físico-químicas específicas ya que la modificación no precisa del contacto directo, lo que evita su contaminación y la alteración de sus propiedades mecánicas. Esta técnica permite escalar el patrón micrométrico elegido al proceso industrial, ya que la impresión del relieve no supone un sobrecoste elevado en relación al valor añadido que le aporta. Una duda que deberá ser resuelta en el futuro respecto a esta metodóloga es su versatilidad para ser aplicada a otro tipo de biopolímeros como la poliimida y el politereftalato de etileno.

En este trabajo hemos podido observar como la variación de la microtopografía interfiere en la adhesión de *S. aureus* a superficies de poliestireno. Lo cual indica que la modificación del relieve de las superficies de los implantes médicos representa una estrategia prometedora para reducir la adhesión de *S. aureus*. Sin embargo, debido a que nuestro conocimiento sobre la influencia de la microtopografía superficial en la adhesión bacteriana sigue siendo en gran medida empírico, es necesario probar experimentalmente cada patrón para determinar su comportamiento en condiciones tanto *in vitro* como *in vivo*.

Si tenemos en cuenta los resultados de nuestro estudio, los patrones más regulares (lineal y de pilares) mejoran la adhesión de una cepa de *S. aureus* formadora de biofilm polisacarídico, mientras que la microtopografía irregular, laminar (LA), reduce la adhesión tanto en condiciones de crecimiento de flujo estático como continuo.

Por otra parte, a la hora de evaluar las posibilidades antiadherentes de una superficie modificada en un modelo de infección hay que tener en cuenta, que una vez que se implanta una superficie en un cuerpo vivo y entra en contacto con los fluidos biológicos, como sangre o suero, las proteínas presentes en los medios cubren inmediatamente el dispositivo médico posiblemente interfiriendo con la adhesión de *S. aureus* y la formación de biofilm (Campoccia *et al.*, 2006; Patel *et al.*, 2007; Hasan *et al.*, 2013; Foster *et al.*, 2014). En este sentido hemos podido observar que la superficie laminar presenta una menor adhesión de *S. aureus* tanto cuando se encuentra en presencia de proteínas del suero humano como cuando se implanta previamente contaminada. Estos resultados sugieren que las superficies de PS con esta topografía podrían proporcionar una prometedora estrategia para reducir la adhesión de *S. aureus* a superficies biomédicas. Otro aspecto que ha quedado pendiente en este trabajo es la evaluación del comportamiento del relieve laminar frente a cepas de *S. aureus* cuya matriz es de naturaleza proteica, así como extender el análisis a otras bacterias patógenas formadoras de biofilms, tanto gram positivas como gram negativas.

2)

En el **Capítulo II** hemos realizado un estudio sistemático para determinar el papel de cada TCS en la colonización y la supervivencia de *S. aureus* en un modelo de infección de catéter subcutáneo murino. En este trabajo se han identificado tres TCS que controlan la colonización en el dispositivo implantado. El descenso en la colonización mostrado por los TCS Agr y SrrAB necesita estudios adicionales para determinar los mecanismos de regulación que están ocurriendo en este modelo de infección. Hemos observado que el descenso en la colonización del catéter en el mutante en *arlRS* está asociado a la deficiencia en la expresión del exopolisacárido PNAG. El papel determinante del PNAG en la colonización de catéteres sugiere, que la inhibición de la síntesis del exopolisacárido PNAG o el empleo de vacunas que induzcan una respuesta inmune frente a este exopolisacárido puede ser una buena estrategia a desarrollar para combatir este tipo de infecciones. En este sentido es importante señalar que los exopolisacáridos son uno de los componentes vacunales mas eficaces para el desarrollo de respuestas protectoras frente a patógenos. Sin embargo, una dificultad que presentan a la hora de utilizarlos como componente vacunal es su enorme diversidad, lo que obliga a utilizar muchos tipos de exopolisacáridos para obtener una protección eficaz frente a un mismo patógeno. En el caso del exopolisacárido PNAG la situación es diferente, porque es producido por muchísimos tipos de patógenos diferentes que van desde bacterias gram positivas como *S. aureus* y *Listeria monocytogenes* a gram negativas como *Aggregatibacter actinomycetemcomitans*, *Burkholderia*, y *E. coli*, a levaduras y protozoos (Cywes-Bentley *et al.*, 2013). Esta circunstancia sugiere la existencia de un proceso de convergencia evolutiva que ha favorecido la producción de este polisacárido. Como consecuencia, el grupo de G. Pier ha propuesto la utilización del exopolisacárido PNAG como un antígeno vacunal universal que permitiría inducir una respuesta inmune protectora universal frente a muchos tipos diferentes de patógenos (Cywes-Bentley *et al.*, 2013).

Otra posibilidad interesante que se abre con nuestros resultados, es el desarrollo de compuestos que bloqueen la actividad de ArlRS y en consecuencia disminuya la capacidad de la bacteria para producir infecciones asociadas a implantes. La identificación de compuestos que bloqueen los TCS es una idea recurrente apoyada por el hecho de que los TCS no están presenten en las células de mamíferos, lo que facilita que su administración resultase inocua para nuestras células (Cegelski *et al.*, 2008; Hansen *et al.*, 2014). Siguiendo este razonamiento, actualmente estamos realizando un escrutinio de compuestos con capacidad para unirse a la región sensora extracelular de

ArlS, y hemos identificado dos candidatos que presentan esta propiedad y están siendo analizados en otra tesis del grupo. En el futuro queremos identificar si su capacidad de unión es suficiente para bloquear la actividad de *arlRS* y evitar la colonización de catéteres en modelos de infección experimental. Otro candidato que despierta un enorme interés para el desarrollo de nuevos antimicrobianos es el sistema de dos-componentes esencial para la viabilidad de *S. aureus* WalKR.

3)

Desde hace varias décadas se está tratando de desarrollar una vacuna eficaz frente a las infecciones estafilocócicas. Desafortunadamente todos estos intentos, algunos de los cuales han llegado hasta etapas avanzadas en fase clínica, han fracasado. En la actualidad, sólo una vacuna multivalente (Pfizer's SA4Ag), que contiene los antígenos ClfA y MntC y los polisacáridos capsulares 5 y 8, y dos anticuerpos monoclonales están siendo evaluados clínicamente en grupos diana con alto riesgo de infección por *S. aureus*. Esto es debido a la variedad de factores de virulencia, toxinas y superantígenos que *S. aureus* expresa diferencialmente durante las distintas etapas del proceso infeccioso o en los distintos tipos de infecciones (agudas o en forma de biofilm).

En el estudio recogido en el **Capítulo III** hemos desarrollado un extracto vacunal para combatir las infecciones causadas por biofilms de *S. aureus*. Durante una infección por biofilm, la matriz extracelular que rodeada a las bacterias es la principal línea de defensa a la que se deben enfrentar las células del sistema inmune para combatir la infección. En este estudio se analizó el potencial de las proteínas secretadas a la matriz del biofilm como vacuna multivalente frente a una infección por biofilm de *S. aureus* utilizando un modelo murino de formación de biofilm sobre mallas de polipropileno. La inmunización con el extracto de la matriz del biofilm dio lugar a una reducción significativa en el número de bacterias del biofilm, así como en el tejido circundante al biofilm formado sobre mallas de polipropileno. Además, los ratones inmunizados mostraron una disminución en la colonización de órganos por las bacterias liberadas del biofilm en la etapa de dispersión.

Teniendo en cuenta los resultados obtenidos en este capítulo de la tesis, el extracto de proteínas de la matriz del biofilm podría tener potencial como vacuna profiláctica que podría dispensarse en pacientes con riesgo de infección por *S. aureus*, como, por ejemplo, los pacientes inmunocoprometidos ingresados en UCI o pacientes sometidos a cirugías de implantación de prótesis u otros dispositivos médicos. La inducción de citoquinas, y en concreto las interleuquinas IL-17 y IL-10, en combinación con el uso de antibióticos podría disminuir la colonización y el posterior desarrollo de biofilms de *S. aureus* sobre la superficie de los dispositivos.

Sin embargo, para poder dar este paso, nos queda todavía pendiente evaluar la contribución de cada una de las proteínas presentes en el extracto de proteínas de la matriz, y la selección de aquellas proteínas con mayor poder inmunoestimulador. Esta identificación nos permitirá definir una combinación de antígenos que proporcione una protección más eficaz, así como su producción de forma recombinante. Esto

homogenizaría la calidad del extracto vacunal y evitaría la existencia de diferencias entre distintos lotes de extractos proteicos, facilitando la estandarización de la producción de la vacuna y la reducción de su coste económico. La rentabilidad económica de una vacuna que previniese las infecciones asociadas a implantes por *S. aureus* estaría fuera de toda duda si su aplicación redujese significativamente el número de infecciones estafilocócicas asociadas a los mismos.

REFERENCIAS

Campoccia, D., Montanaro, L., Agheli, H., Sutherland, D.S., Pirini, V., Donati, M.E., and Arciola, C.R. (2006) Study of Staphylococcus aureus adhesion on a novel nanostructured surface by chemiluminometry. *The International Journal of Artificial Organs* **29**: 622–629.

Cegelski, L., Marshall, G.R., Eldridge, G.R., and Hultgren, S.J. (2008) The biology and future prospects of antivirulence therapies. *Nat Rev Microbiol* **6**: 17–27.

Corvec, S., Portillo, M.E., Pasticci, B.M., Borens, O., and Trampuz, A. (2012) Epidemiology and new developments in the diagnosis of prosthetic joint infection. *The International Journal of Artificial Organs* **35**: 923–934.

Cywes-Bentley, C., Skurnik, D., Zaidi, T., Roux, D., Deoliveira, R.B., Garrett, W.S., *et al.* (2013) Antibody to a conserved antigenic target is protective against diverse prokaryotic and eukaryotic pathogens. *Proc Natl Acad Sci USA* **110**: E2209–18.

Foster, T.J., Geoghegan, J.A., Ganesh, V.K., and Höök, M. (2014) Adhesion, invasion and evasion: the many functions of the surface proteins of Staphylococcus aureus. *Nat Rev* Microbiol **12**: 49–62.

Hansen, J., Mailand, E., Swaminathan, K.K., Schreiber, J., Angelici, B., and Benenson, Y. (2014) Transplantation of prokaryotic two-component signaling pathways into mammalian cells. *Proc Natl Acad Sci USA* **111**: 15705–15710.

Hasan, J., Crawford, R.J., and Ivanova, E.P. (2013) Antibacterial surfaces: the quest for a new generation of biomaterials. *Trends in Biotechnology* **31**: 295–304.

Patel, J.D., Krupka, T., and Anderson, J.M. (2007) iNOS-mediated generation of reactive oxygen and nitrogen species by biomaterial-adherent neutrophils. *J Biomed Mater Res A* **80**: 381–390.

CONCLUSIONES

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1. La modificación del micro-relieve de la superficie de los implantes tiene un impacto en la capacidad de adhesión de *S. aureus*, sugiriendo que se trata de una estrategia que se debe tener en cuenta para reducir el desarrollo de biofilms en la superficie de los dispositivos médicos. Con nuestro conocimiento actual del proceso, nuestros resultados también indican la imposibilidad para predecir las consecuencias que un determinado relieve va a tener en la adherencia de la bacteria, siendo necesario el análisis experimental para conocer dicho comportamiento.
2. El relieve laminar ha sido la topografía que ha tenido un mejor comportamiento antiadherente frente a *S. aureus* en condiciones de flujo estático y continuo, tanto en presencia como en ausencia de proteínas del suero. Dicho relieve también disminuye la adherencia de *S. aureus* en un modelo murino de formación de biofilm sobre materiales implantados intraperitonealmente.
3. El análisis global de la familia de 16 TCS de *S. aureus* ha mostrado que la mutación de los sistemas *arlRS*, *srrAB* y *agr* disminuye la adhesión de la bacteria a catéteres implantados en un modelo de infección subcutáneo en ratón. La deficiencia del resto de los sistemas no causa una disminución significativa de la capacidad de *S. aureus* de colonizar la superficie del catéter.
4. La deficiencia del mutante *arlRS* en la adherencia a los catéteres se debe a una disminución en la expresión del exopolisacárido PNAG, causada por una mayor actividad transcripcional del represor IcaR.
5. Independientemente de la naturaleza de la matriz extracelular del biofilm de *S. aureus*, existe un núcleo común de proteínas que se secretan y forman parte de dicha matriz.
6. La inmunización de ratones con un extracto de exoproteínas de la matriz del biofilm de *S. aureus* induce la producción de anticuerpos con actividad opsonofagocítica y de interleuquinas IL-10 e IL-17.
7. La respuesta inmune generada por la inmunización con un extracto de exoproteínas de la matriz del biofilm protege a ratones de infecciones causadas por biofilms de *S. aureus*, utilizando un modelo murino de infección de malla intraperitoneal. Los ratones inmunizados presentan una reducción en el número de bacterias formando parte del biofilm y una colonización moderada del tejido circundante y de los riñones por bacterias que se liberan a partir del biofilm.

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1. The modification of the micro-topography of implant surfaces has an impact on the adhesion capacity of *S. aureus*, suggesting that this is a strategy that must be taken into account to reduce the development of biofilms on the surface of medical devices. With our current knowledge of the process, our results also indicate the impossibility to predict the consequences that a certain topography will have on bacterial adherence, with experimental analysis being necessary to know this behavior.
2. The laminar topography has been the topography showing the best antiadherent behavior against S. aureus under static and flow conditions, both in the presence and in the absence of serum protein. Such topography also decreases the adherence of S. aureus in a murine model of biofilm formation on materials implanted intraperitoneally.
3. The global analysis of the 16 TCS family of S. aureus has shown that the mutation of arlRS, srrAB and agr systems, decreases the adhesion of the bacteria to implanted catheters in a murine subcutaneous infection model. The deficiency of the rest of the systems does not cause a significant decrease in the ability of S. aureus to colonize the surface of the catheter.
4. The deficiency of the arlRS mutant in adherence to catheters is due to a decrease in the expression of the exopolysaccharide PNAG, caused by a greater transcriptional activity of the IcaR repressor.
5. Regardless of the nature of the S. aureus biofilm extracellular matrix, there is a common core of proteins that are secreted and are part of such matrix.
6. Immunization of mice with an extract of exoproteins from the S. aureus biofilm matrix induces the production of antibodies that have opsonophagocytic activity and interleukins IL-10 and IL-17.
7. The immune response generated by immunization with an exoprotein extract from the biofilm matrix protects mice from infections caused by S. aureus biofilms, using a murine model of intraperitoneal mesh infection. Immunized mice show a reduction in the number of bacteria inside the biofilm and a moderate colonization of the surrounding tissue and kidneys by bacteria that are released from the biofilm.